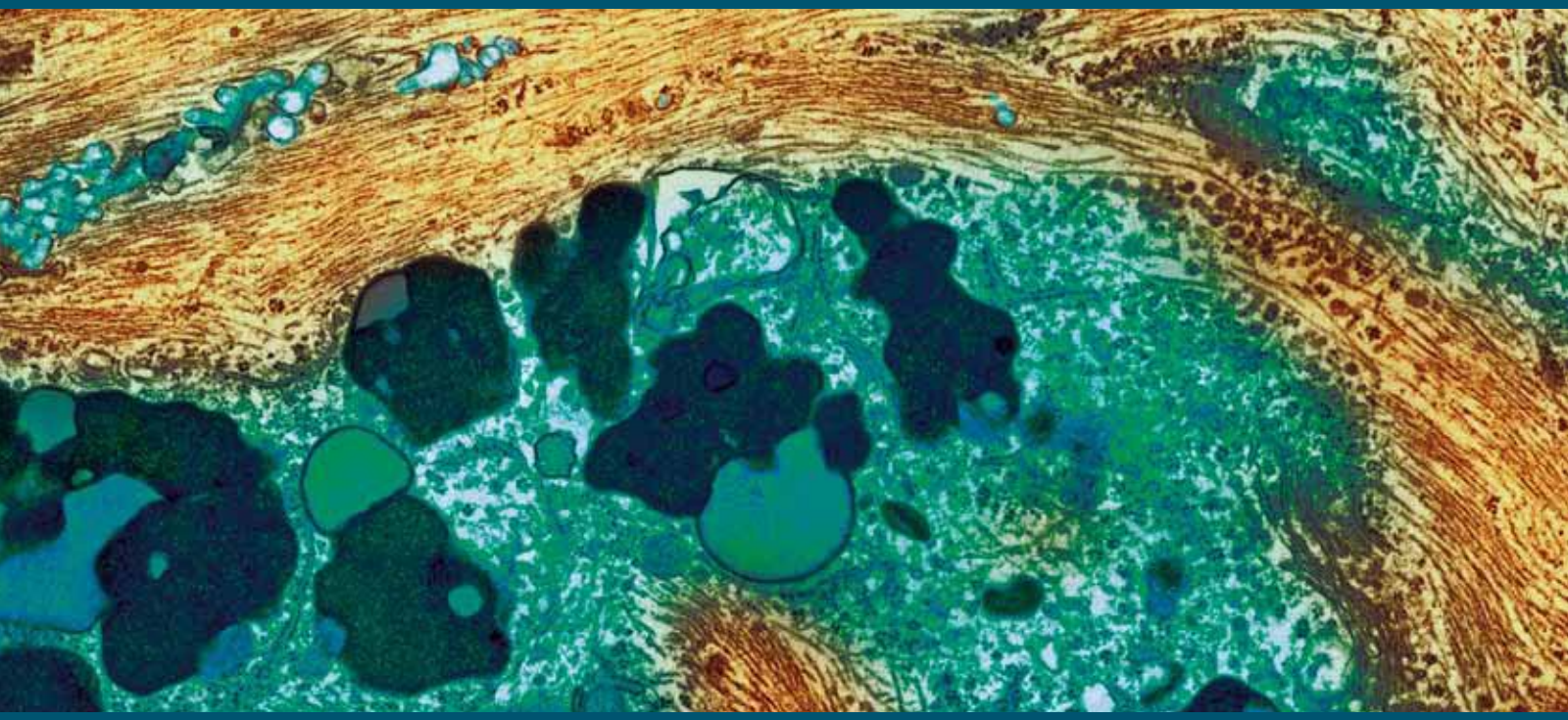
International Journal of Alzheimer's Disease

Copper Status in Alzheimer's Disease and Other Neurodegenerative Disorders: Genetics, Mechanisms, Neurophysiology, and Therapies

Guest Editors: Rosanna Squitti, D. Larry Sparks, Tjaard U. Hoogenraad, and George J. Brewer



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Editorial

Copper Status in Alzheimer's Disease and Other Neurodegenerative Disorders: Genetics, Mechanisms, Neurophysiology, and Therapies

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The present special issue is an attempt to explore some of the diverse aspects of copper involvement in the physiology of the brain. The contributions examine very hot topics, such as the relationship between copper and synaptic transmission or copper involvement in Alzheimer's disease (AD) and even include a computational model of copper binding to the amyloid precursor protein (S. Azini and R. Rouk 2011). In this model, the authors applied theoretical methods of computational chemistry to determine structure, binding affinities, and reduction potentials of Cu(II) and Cu(I) bound to models of the Asp1, Ala2, His6, and His13His14 regions of the amyloid beta peptide.

A bulk of new studies has been recently published on the role of copper in the biological processes subtending synaptic transmission, and three contributions on this subject are included in the special issue. Specifically, one contribution describes a study in a cellular model of oocytes, which investigated the connection between copper and the prion protein in neurotransmission mediated by ATP-evoked currents (R. A. Lorca et al., 2011). A study on mouse hippocampal slices investigates copper inhibition of glutamatergic NMDA receptor-independent long-term potentiation (N. L. Salazar-Weber and J. P. Smith 2011), and a human model explores the effects of systemic copper on glutamatergic mediated excitability in the primary somatosensory cortex (F. Tecchio et al., 2011).

Since the original papers that have been fuelling a heated debate in the literature about the pros and cons of copper on cognition are rather scattered and discordant, the aim of the editors was to select contributions that all together would provide the reader with an adequate overview of the issue but would still leave the reader free to form personal and hopefully critical opinions. In this section, some of the papers give a good overview of the nonunivocal *in vitro*, animal and human findings which demonstrate the involvement of copper in AD (D. Kaden et al. 2011). Also, in their paper, Y. Manso and colleagues (Y. Manso et al., 2011) review the preclinical and clinical literature dedicated to therapeutic interventions aimed at delaying or possibly reverting AD progression via the modulation of brain copper levels (Y. Manso et al., 2011). In the same section, some reports are preliminary but still original in their content, as, for example, the paper dealing with the influence of water quality on the cholesterol-fed rabbit model, which suggests that the origin of the brain neurofibrillary tangles is possibly systemic and somehow related to disturbances to the blood brain barrier mediated by copper ingested through the diet (D. L. Sparks et al., 2011), as well as the

study by S. Bucossi and coworkers (S. Bucossi et al., 2011) which demonstrates an association between single nucleotide polymorphisms of the Wilson's disease gene and AD. T. U. Hoogenraad discusses further the link between these two neurological disorders (T. U. Hoogenraad 2011), which share the presence of abnormalities in toxic nonceruloplasmin (also named "free") copper, proposing Zinc therapy as a valid tool against AD, as it has already proven to be in Wilson's disease. The perspective that comes out of his contribution (T. U. Hoogenraad 2011), for some maybe visionary or prophetic, is probably somehow adherent to the author's attitude, pragmatic, as can be expected from a physician who has dedicated his career to Wilson's disease clinical care, but also filled with enthusiasm, as demonstrated by his attitude to be prone to ideas of shifting paradigms: he was one of the pioneer of Zinc therapy in Wilson's disease.

With a totally different approach, G. J. Brewer (G. J. Brewer 2011) discusses AD from an epidemiologic point of view, advancing the hypothesis that ingestion of inorganic copper from copper-made plumbing or via consumption of copper-rich dietary supplements may have a role in AD generation. Finally, the issue presents also a study connecting higher levels of copper to gender differences and cognitive status (J. F. Quinn et al., 2011).

In conclusion, is copper good or bad in general for human health and in particular for cognition? Of course, the question is far from being answered in this special issue, but it is well discussed. These editors recommend reading carefully the diverse interpretations presented but also going back and read the original works which are at the basis of them. In fact, even though reliable, review articles often decrease the critical attitude of the reader, as recent publications about citation bias have pointed out [1].

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George J. Brewer

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[1] M. Schrag, C. Mueller, U. Oyoyo, M. A. Smith, and W. M. Kirsch, "Iron, zinc and copper in the Alzheimer's disease brain: a quantitative meta-analysis. Some insight on the influence of citation bias on scientific opinion," *Progress in Neurobiology*, vol. 94, no. 3, pp. 296–306, 2011.

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Research Article

On the Involvement of Copper Binding to the N-Terminus of the Amyloid Beta Peptide of Alzheimer's Disease: A Computational Study on Model Systems

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Density functional and second order Moller-Plesset perturbation theoretical methods, coupled with a polarizable continuum model of water, were applied to determine the structures, binding affinities, and reduction potentials of Cu(II) and Cu(I) bound to models of the Asp1, Ala2, His6, and His13His14 regions of the amyloid beta peptide of Alzheimer's disease. The results indicate that the N-terminal Asp binds to Cu(II) together with His6 and either His13 or His14 to form the lower pH Component I of Aβ. Component II of Aβ is the complex between Cu(II) and His6, His13, and His14, to which an amide O (of Ala2) is also coordinated. Asp1 does not bind to Cu(II) if three His residues are attached nor to any Cu(I) species to which one or more His residues are bound. The most stable Cu(I) species is one in which Cu(I) bridges the N_δ of His13 and His14 in a linear fashion. Cu(I) binds more strongly to Aβ than does Cu(II). The computed reduction potential that closely matches the experimental value for Cu(II)/Aβ corresponds to reduction of Component II (without Ala2) to the Cu(I) complex after endergonic attachment of His6.

1. Introduction

The sequence of the human amyloid beta peptide, Aβ(1-42), in single-letter code, is:

$$D_1AGFRH_6DSGY_{10}EVH_{13}H_{14}QKLVFFAED\text{-}VGSNKGAIIGLM_{35}VGGVVIA_{42}$$

Cupric ion (Cu^{2+}) forms a 1 : 1 complex with Aβ(1-40) or Aβ(1-42) [1] with approximately picomolar or nanomolar affinity (K_d^{cond}=1 × 10^{-11} M for Aβ(1-42); [1] K_d^{cond} = 57 ± 5 × 10^{-9} M for Aβ(1-40) [2]). The presence of added or *in vivo* buffers may lower the effective affinity to a considerable extent [1, 3]. The Cu(II) is bound as type 2, that is, distorted square planar arrangements of ligands with possibly one or two additional axial ligands. EPR measurements indicate a 3N1O equatorial coordination pattern [4]. At room temperature, two [3, 5–7] or three [8] forms whose populations are dependent on pH are observed. The lower pH component is referred to as component I (or Ia and Ib) [9] while the higher pH component is Component II, all with 3N1O coordination. It is likely that the three N ligands are due to a combination of the imidazole groups of His6, His13, His14, a deprotonated amide N, and the N-terminus since these are the most common ligands for copper bound in proteins. Indeed, there is direct evidence, obtained by ESR experiments on Aβ(1-16) in which the histidines were isotopically enriched with ^{15}N, that all three His residues bind to Cu(II) at physiological pH = 7.4 [10–12]. The origin of the O ligand is a subject of some debate, although Tyr10, Glu3, Asp7, and Glu11 have been ruled out, as has water [13]. On the basis of hyperfine sublevel correlation (HYSCORE) spectroscopy applied to site-specific ^{13}C and ^{15}N labeled Aβ(1-16), the carbonyl of Ala2 is implicated as the O ligand in the coordination mode at higher pH = 8.0 (Component II), along with all three His residues [12]. This result is supported by computational modeling of Aβ(1-16) [14]. At higher pH (pH = 8.7–9), studies by both EPR [6] and NMR [7] on labeled compounds seem to indicate that deprotonation and coordination of the amide N of Ala2 occurs, together with the N-terminal NH_2 and one or two

His side chains. Hureau and Faller and coworkers [6, 7] assigned this high pH structure as "Component II," but it is at odds with the structure deduced by others at physiological pH that does not involve the N-terminus and was designated Component II.

There is general but not universal agreement that the N-terminus is also part of the native Aβ/Cu binding site, although the nature of its involvement is still unclear. In an earlier study, Karr and coworkers found that copper binding to Aβ was sensitive to changes in the N-terminus, including deletion [4], but, in a subsequent study, they note that removal or mutation of Asp1 does *not* disrupt the equatorial coordination sphere [13]. They propose that the N-terminus participates via hydrogen bonding to an axial ligand [13]. Kowalik-Jankowska et al. carried out potentiometric and spectroscopic measurements on both human and mouse Aβ(1-16) and Aβ(1-28) over a wide pH range, 2.5–10.5, and noted a significant shift in the coordination pattern upon acetylation of the N-terminus in all cases [15]. Barnham and coworkers used multifrequency CW-EPR spectroscopy applied to site-specific ^{15}N-labeling at Asp1, His6, His13, and His14 of Aβ(1-16) to deduce the presence at pH = 6-7 of two independent 3N1O Cu^{2+} coordination modes both of which incorporated the N-terminal NH_2 group, an O atom, His6, but only one of His13 (Component Ia) or His14 (Component Ib) [9]. On the other hand, Hong et al., propose that the 3N1O coordination arises from an equilibrium between *three* structures at pH = 6.5–7.4 that all incorporate, beside an "O" atom, the N-terminal NH_2 group, His6, and either a deprotonated backbone amide residue or one of His13 or His14, but not simultaneously His13 and His14 [8]. Both low pH results are in qualitative agreement with the structure assigned to Component I at pH = 6 by Hureau and Faller and coworkers who proved coordination of the NH_2 and carbonyl groups of Asp1 together with His6 and one of His13 and His14.

Besides being highly pH dependent, indications are that copper coordination is probably size dependent as well. NMR studies of Cu^{2+}(aq) interacting with full-length uniformly ^{15}N-labelled Aβ(1-40) at pH = 7.3 found that the Asp1 signals were not shifted upon addition of Cu^{2+} and seem to indicate that the N-terminus is not involved in copper binding at all [16].

On the basis of ab initio computations on model systems, we have previously proposed that the primary binding site of Cu(II) to Aβ is His13His14 which provide two of the three observed N ligands through N_δ of the imidazole groups. By our procedures, we could not verify the presence or absence of the carbonyl O atom of Ala2 as has been proposed experimentally. However, in the computations, the O of the intervening amide group (O of His13) inevitably became coordinated to the Cu(II) [17]. We proposed that this amide oxygen corresponds to the mysterious "O" of the 3N1O coordination pattern and that the third "N" was probably that of His6 or Asp1. Possibly because of the excessively high affinity constant calculated in that study, $\log_{10}K_{\text{aff}} = 19$, this suggestion has not been taken seriously by any of the experimental groups that have examined Cu coordination to Aβ. We have since reevaluated the binding by the procedures employed in the present work and found a substantially lower value, $\log_{10}K_{\text{aff}} = 6.3$, [18] that is more in accordance with the accepted data for Cu(II) binding to Aβ [2]. We note that isothermal calorimetry experiments by Hong et al. found that Cu^{2+} binding to His13His14 is most favored by the enthalpic contribution to the free energy change but that the enthalpic preference was overwhelmed by an unfavorable entropic term [8].

Less is known experimentally about the coordination of Cu(I) to Aβ. We have previously [19, 20] modeled the attachment of Cu(I) to the His13His14 region of Aβ by computational methodology similar to that employed in the present work. The computations suggest that Cu(I) is dicoordinated and bound to the two His sidechains via the proximal N atoms (N_δ) in a linear fashion. Himes et al. have demonstrated such binding experimentally by EXAFS and XANES data and results carried out on copper(I) complexes of small HisHis peptides [21], and fragments of Aβ [22]. Such a structure was also found by XAS spectroscopy on Aβ(1-40) [23]. Apparently, Cu(I) binds to Aβ(1-16) with femtomolar affinity [24], much higher than does Cu(II). The linear two-coordinate complexes were also able to add a third imidazole ligand in a T-shaped configuration in a dynamic process that interchanges all three His residues [25]. Interestingly, the two-coordinate complexes were resistant to oxidation, but the three-coordinate complexes were redox active [26]. However, the Cu(I) complexes with HisHis containing fragments were able to produce H_2O_2 in the presence of O_2 but without added reducing agents. The amount of H_2O_2 produced was independent of the presence of Tyr10 (a potential source of electrons) or other residues such as Asp1 or His6 which are potential binding sites for the copper [22].

The reduction potential of Cu(II)/Aβ has been investigated by several groups. Aqueous Cu^{2+} has a one-electron reduction potential $E^\circ = 0.17$ V versus the standard hydrogen electrode (SHE). An early report that monomeric Cu(II)/Aβ(1-42) had an exceptionally high reduction potential $E^\circ \approx 0.7$ V versus SHE [27] has been discounted as due to oligomer formation. The consensus value is E°("Cu(II)/Aβ"/"Cu(I)/Aβ") $\approx$ 0.30 V–0.34 V [28, 29] which represents a modest elevation of the oxidizing power of the Cu(II) upon complexation to Aβ. Brzyska et al., find a concentration- and buffer-dependent reduction of the reduction potential upon addition of Cu(II) to monomeric Aβ(1-40), although, in the absence of buffer, their value is similar to the others [30]. As noted above, there is uncertainty as to the actual nature of the oxidized and reduced species involved. Guilloreau et al. report a wide difference between the reduction potential and oxidation potential of copper bound to Aβ(1-28), 0.33 V and 0.63 V, respectively [29]. This is taken as an indication of different geometries at the Cu(II) and Cu(I) binding sites [30].

We have undertaken the present study in order to examine the role of Asp1 in the coordination of Cu(II) as well as Cu(I) in Aβ. We employ a higher level of theoretical treatment than was previously applied in order to estimate the binding affinities of complexes containing all combinations of Asp1, modeled by the N-methyl

amide derivative, **1** (Figure 2), His6, modeled by imidazole (**Im**), an amide carbonyl, modeled by N-methylacetamide (NMA), and His13His14, modeled as previously [17, 20] by *N*-*α*-dihydrourocanylhistamine, **5** (Figure 3). Simultaneous involvement of both Asp1 and Ala2 were modeled by the N-methyl amide derivative of Asp1Ala2, **3** (Figure 1). Reduction potentials for various Cu(II)/Cu(I) couples were also derived and compared with experimental values in order to elucidate the nature of the species involved.

2. Computational Methods

All calculations were carried out with Gaussian 03 and 09 [31, 32] using the hybrid density functional method, B3LYP [33], and second-order Moller-Plesset perturbation theory. Gaseous-phase geometry optimization, harmonic frequency calculation, and thermochemical parameters were determined at the B3LYP/6-31 + G(d) basis set, which is henceforth referred to as the small basis set (SB). The frequency calculation confirmed that the optimized structures were at local minima on the potential energy hypersurface. The zero point energies were scaled by 0.9806 [34]. However, this was not done for the thermal correction of enthalpy or entropies. In the case of the zwitterionic Asp1, **1** (Figure 1) and Asp1Ala2, **3** (Figure 2), it was necessary to optimize the structure and carry out frequency analysis in the presence of the solvent reaction field (SCRF = IEFPCM), where IEFPCM is the integral equation following polarizable continuum model [35, 36] with the default parameters for water. Some structures had a great deal of conformational flexibility. Chemical intuition was used to seek the most stable structures, and no attempt was made to do a comprehensive search of conformational space. Instead, account was taken of conformational flexibility by the addition of an approximate entropy of mixing term, $R\ln(n)$, where n is an estimate of the number of conformers derived by simple rotamer counting [37]. Values of n are listed in Table S2 in Supplementary Materials available online at doi: 10.4061/2011/539762. Entropies were also converted to a state of 1 M by addition of the term for volume change, $R\ln(1/24.46)$, where 24.46 litres is the volume of 1 mol of ideal gas at 298 K. For more accurate enthalpies and to compensate for the lack of long-range dispersion energy in B3LYP, single-point energies were calculated at an approximation for the MP2/LB level,

$$E(\text{MP2/LB}) \approx E(\text{MP2/SB}) + E(\text{B3LYP/LB}) - E(\text{B3LYP/SB}), \quad (1)$$

where LB is the large basis set, 6-311+(2df,2p).

Details of all computed quantities and structural information, are provided in Tables S1 and S2, respectively. Molden 4.0 was used as a visualization tool [38].

2.1. Free Energies of Solvation, ΔG_{solv}, and Empirical Corrections. In order to calculate the free energy change in water, $\Delta G_{(\text{aq})}$, the change in the free energy of solvation, $\Delta\Delta G_{\text{solv}}$, was added to the free energy change in the gaseous phase, $\Delta G_{(\text{g})}$, corrected for a standard state of 1 M. ΔG_{solv} was determined using IEFPCM [35, 36] as implemented in G03, and the B3LYP/SB density. In our experience, charged species are undersolvated by the IEFPCM with standard scaling of the united atom Hartree-Fock (UAHF) radii, so selective scaling was applied as follows: the radii of the metal ion and all atoms directly attached to it were scaled by a factor of 1.1; all other atoms were scaled by the default value, 1.2. Experimental rather than calculated relative free energies of solvation were applied where available in order to reduce errors further. For the proton, $\Delta G_{\text{solv}}(\text{H}^+) = -1107\,\text{kJ mol}^{-1}$ was adopted [39]. The experimental value of ΔG_{solv} was adopted for H_2O, $-16.2\,\text{kJ mol}^{-1}$, where the value reflects the fact that water is 55.6 M. For all other species, free energies of solvation were taken as calculated by the procedure described above.

2.2. Calculation of Reduction Potentials for "Cu(II)"/"Cu(I)" Redox Couples. The mechanisms proposed below involve only intermolecular single-electron transfer processes. The standard reduction potential of a "Cu(II)"/"Cu(I)" couple, relative to the standard hydrogen electrode (SHE), $\mathbf{E}^\circ$("Cu(II)"/"Cu(I)"), is defined by

$$E^\circ(\text{"Cu(II)"/"Cu(I)"}) = -\left(\Delta G^{\text{Cu}}_{(\text{aq})} - \Delta G^{\text{SHE}}_{(\text{aq})}\right)/F, \quad (2)$$

where F is the Faraday constant, $F = 96.485\,\text{kJ mol}^{-1}\,\text{V}^{-1}$, $\Delta G^{\text{SHE}}_{(\text{aq})}$ is the free energy change for the standard hydrogen cell half reaction, $(1/2)\text{H}_{2(\text{g})} + \text{e}^- \rightarrow \text{H}^+_{(\text{aq})}$, ($\Delta G^{\text{SHE}}_{(\text{aq})} = -418\,\text{kJ mol}^{-1}$, ignoring the electron) [40], and $\Delta G^{\text{Cu}}_{(\text{aq})}$ is the calculated free energy change for reaction (3), again ignoring the electron

$$\text{"Cu(II)"}_{(\text{aq})} + \text{e}^- \longrightarrow \text{"Cu(I)"}_{(\text{aq})} + a\text{L}. \quad (3)$$

In (3), "Cu(II)" and "Cu(I)" represent species containing oxidized and reduced copper, respectively. The symbol aL recognizes the fact that a number of ligands may be shed in the reduction process and that the associated entropy change may be an important component of the free energy change. The actual potential, E, of the half reaction under ambient conditions is related to the standard potential, E°, through the Nernst equation,

$$E = E^\circ - \left(\frac{RT}{F}\right)\ln Q, \quad (4)$$

where Q is the reaction quotient specifying concentrations of oxidized and reduced components and other species associated with the chemical change. In the special case that n protons are consumed in solution buffered at pH = 7 under otherwise standard conditions, the reaction quotient reduces to $Q = 10^{7n}$, and the symbol, $E^{\circ\prime}$, denotes the potential at pH 7 ($E^{\circ\prime} = E^\circ - (RT/F)\ln Q = E^\circ - 0.41\text{nV}$).

For most of the energy differences calculated in the following sections, errors inherent in the calculation of absolute values could be expected to cancel yielding reliable relative energies. However, this is less likely to be the case for the calculation of aqueous free energy changes for reactions such as (3). Since a transition element is involved and the number of electrons changes, the enthalpy change will be less accurately described at this theoretical level than expected for lighter elements. An extreme case is illustrated

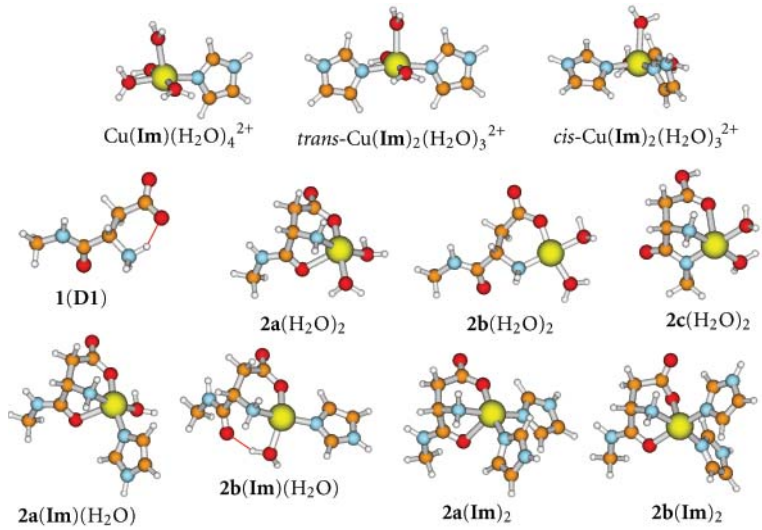

Figure 1: Structures with Cu(II), Asp1, and imidazole **Im**. Ball colors: Cu: large yellow; C: orange; N: blue; O: red; H: white.

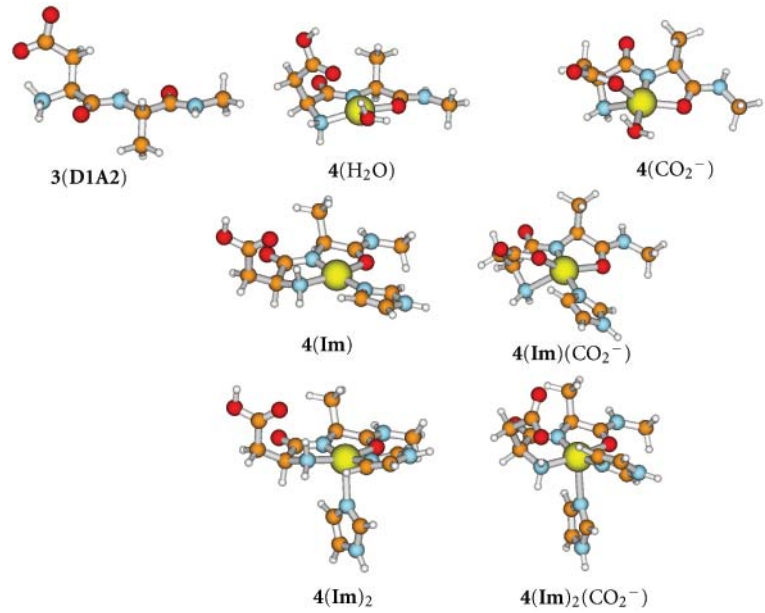

Figure 2: Structures with Cu(II), Asp1Ala2 **3**, and imidazole **Im**. Ball colors: Cu: large yellow; C: orange; N: blue; O: red; H: white.

by the difference between the calculated (at the B3LYP/LB level) and experimental second ionization potential of atomic copper (IE2 (calc) = 2008 kJ mol^{-1}; IE2 (exp) = 1958 kJ mol^{-1} [41]). The discrepancy is most likely due to the unequal treatment of electron correlation (an enthalpic term). As in a previous publication [42], we assume that the error in the ionization potential of Cu^+ will be present in the reduction potentials, E°("Cu(II)"$_{(aq)}$/"Cu(I)"$_{(aq)}$), irrespective of the metal environment since they all involve the change in copper oxidation state from +2 to +1. Without correcting for the error in the enthalpy change, the calculated value for the reduction of aqueous cupric ion is $E^\circ(Cu(H_2O)_5{}^{2+}/Cu(H_2O)_3{}^{+}) = 0.42$ V versus SHE, compared to the experimental value, $E^\circ(Cu^{2+}(aq)/Cu^{+}(aq)) = 0.17$ V [41]. An empirical correction of +57 kJ mol^{-1} brings the calculated and experimental numbers into agreement. Thus for addition of an electron to any Cu^{2+} species, the correction to the calculated ΔH is taken as +57 kJ mol^{-1}.

With the procedures described above, we expect that aqueous free energy changes, $\Delta G_{(aq)}$, will be accurate to ± 15 kJ mol^{-1} for all of the reactions considered.

3. Results and Discussion

The B3LYP/SB-optimized structures and all calculated energies and thermochemical properties are provided in Tables S1 and S2 and shown in Figures 1–3. Chemical transformations and aqueous free energy changes are given in the following

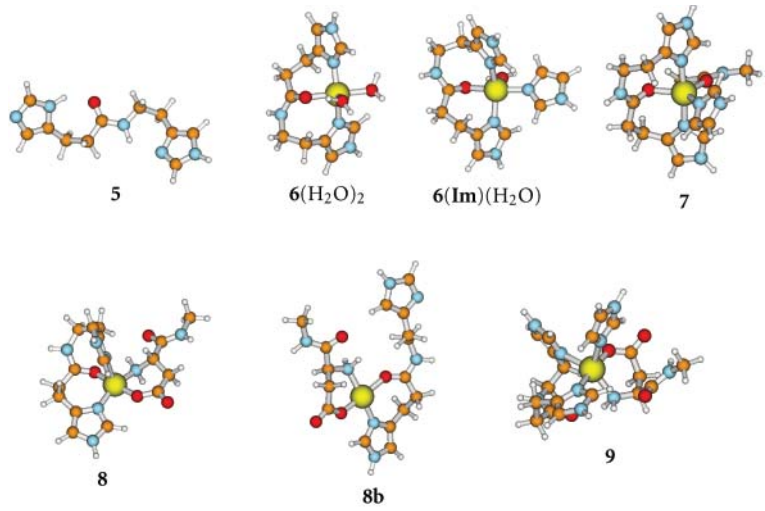

Figure 3: Structures with Cu(II), HisHis **5**, Asp1, and Imidazole **Im**. Ball colors: Cu = large yellow; C = orange; N = blue; O = red; H = white.

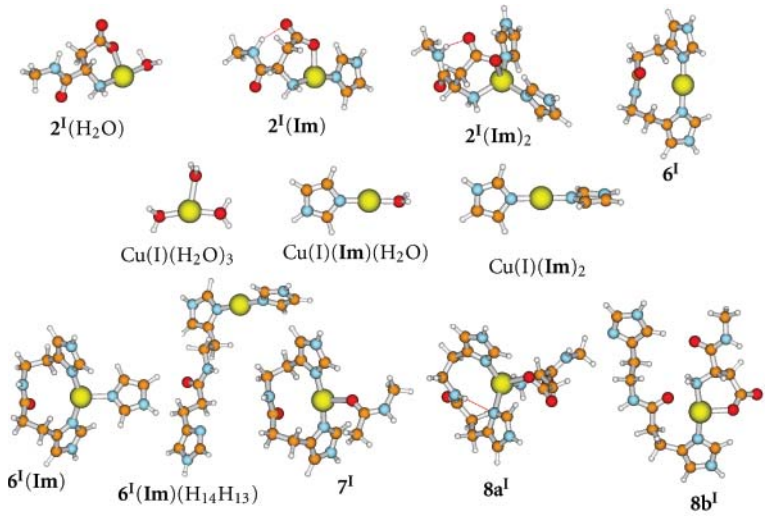

Figure 4: Stable structures with Cu(I). Ball colors: Cu = large yellow; C = orange; N = blue; O = red; H = white.

in the form of numbered equations which are repeated in Table 1 along with a complete breakdown of the components at the MP2/LB level. The corresponding data at the B3LYP/LB level are given in Table S3.

4. Interaction of Cu^{2+}(aq) with the N-Terminal Asp1 **1**

The N-terminal Asp1 of Aβ is modeled by **1** (Figure 1), where the C-terminus is derivatized by $NHCH_3$. The most stable form of **1** in water is zwitterionic. The most stable form of the aqueous cupric ion is the pentaaqua structure, $Cu(H_2O)_5^{2+}$. Reaction with **1** yielded numerous aquated structures **2** with different patterns of chelation:

$$\mathbf{1} + Cu(H_2O)_5^{2+} \longrightarrow \mathbf{2a}(H_2O)_2 + 3H_2O + H^+; \quad \Delta G_{(5a)} = -18.0\,\text{kJ mol}^{-1}, \tag{5a}$$

$$\mathbf{1} + Cu(H_2O)_5^{2+} \longrightarrow \mathbf{2b}(H_2O)_2 + 3H_2O + H^+; \quad \Delta G_{(5b)} = +14.3\,\text{kJ mol}^{-1}, \tag{5b}$$

$$\mathbf{1} + Cu(H_2O)_5^{2+} \longrightarrow \mathbf{2c}(H_2O)_2 + 3H_2O + H^+; \quad \Delta G_{(5c)} = +29.1\,\text{kJ mol}^{-1}, \tag{5c}$$

where $\Delta G_{(5a)}$, $\Delta G_{(5b)}$, and $\Delta G_{(5c)}$, have been adjusted to pH = 7. Structures of **2a**$(H_2O)_2$, **2b**$(H_2O)_2$, and **2c**$(H_2O)_2$ are shown in Figure 1. It should be noted from Table 1 that the release of multiple water molecules in processes such as (5a)–(5c) endows a large entropic component favoring complex formation. In addition, release of a proton into a solution buffered to pH = 7 provides an additional 40 kJ mol^{-1} ($=RT\ \ln(10^{-7})$) as a driving force for the forward direction.

Of the three 1 : 1 complexes, only the most stable, **2a**$(H_2O)_2$, is formed exergonically at physiological pH. This structure has a square pyramidal configuration with three-point coordination of the Asp residue to the cupric ion.

Table 1: Relative energies at 298 K of species discussed in the text: enthalpies based on MP2/LB energies[a].

Process	Eqn. no.	$\Delta H_{(g)}$ (kJ mol^{-1})	$-T\Delta S_{(g)}$ (kJ mol^{-1})	$\Delta G_{(g)}$ (kJ mol^{-1})	$\Delta\Delta G_{solv}$ (kJ mol^{-1})	$\Delta G_{(aq)}$ (kJ mol^{-1})
1 + $Cu(H_2O)_5^{2+}$ → **2a**$(H_2O)_2$ + $3H_2O$ + H^+	(5a)	480.8	−68.6	412.2	−390.2	−18.0
1 + $Cu(H_2O)_5^{2+}$ → **2b**$(H_2O)_2$ + $3H_2O$ + H^+	(5b)	533.6	−76.1	457.6	−403.2	14.3
1 + $Cu(H_2O)_5^{2+}$ → **2c**$(H_2O)_2$ + $3H_2O$ + H^+	(5c)	519.3	−68.8	450.5	−381.4	29.1
3 + $Cu(H_2O)_5$ → **4**(H_2O) + $4H_2O$ + H^+	(6)	464.3	−90.6	373.6	−323.4	10.2
Im + $Cu(H_2O)_5^{2+}$ → Cu(**Im**)$(H_2O)_4^{2+}$ + H_2O	(7a)	−157.3	6.5	−150.8	150.3	−0.5
2**Im** + $Cu(H_2O)_5^{2+}$ → $Cu(\mathbf{Im})_2(H_2O)_3^{2+}$ + $2H_2O$	(7b)	−298.4	14.3	−284.1	273.2	−10.9
2a$(H_2O)_2$ + **Im** → **2a**(H_2O) + H_2O	(8a)	−105.0	3.9	−101.1	77.0	−24.1
1 + Cu(**Im**)$(H_2O)_4^{2+}$ → **2a**(**Im**)(H_2O) + $3H_2O$ + H^+	(8b)	533.1	−71.2	461.9	−463.5	−41.6
2a(**Im**)(H_2O) + **Im** → **2a**$(\mathbf{Im})_2$ + H_2O	(9a)	−85.5	6.1	−79.4	68.4	−11.0
1 + $Cu(\mathbf{Im})_2(H_2O)_3^{2+}$ → **2a**$(\mathbf{Im})_2$ + $3H_2O$ + H^+	(9b)	588.7	−72.9	515.8	−517.9	−42.2
4(H_2O) + **Im** → **4**(**Im**) + H_2O	(10a)	−89.8	3.1	−86.7	66.2	−20.5
3 + $Cu(H_2O)_5^{2+}$ + **Im** → **4**(**Im**) + $5H_2O$ + H^+	(10b)	374.5	−87.5	287.0	−257.3	−10.3
4(**Im**) + **Im** → **4**$(\mathbf{Im})_2$	(11)	−105.6	35.9	−69.7	76.6	6.9
4(**Im**) + **Im** + **1** → **2a**$(\mathbf{Im})_2$ + **3**	(12)	−84.1	28.8	−55.3	12.5	−42.8
5 + $Cu(H_2O)_5^{2+}$ → **6**$(H_2O)_2$ + $3H_2O$	(13)	−300.3	−29.5	−329.8	293.8	−36.0
6$(H_2O)_2$ + **Im** → **6**(**Im**)(H_2O) + H_2O	(14)	−121.9	9.6	−112.2	99.7	−12.5
6(**Im**)(H_2O) + NMA → **7** + H_2O	(15)	68.5	−3.7	64.8	−85.4	−20.5
1 + **6**$(H_2O)_2$ → 8**a** + $2H_2O$ + H^+	(16a)	623.3	−44.8	578.4	−535.6	2.8
2a$(H_2O)_2$ + **5** → **8a** + $2H_2O$	(16b)	−157.9	−5.7	−163.6	148.4	−15.2
1 + **6**(**Im**)(H_2O) → **9** + H_2O + H^+	(17a)	608.0	−8.4	605.8	−543.8	22.0
9 → **8a** + **Im**	(17b)	130.9	−46.0	84.8	−91.5	−6.7
9 + H_2O + H^+ → **5** + **3a**(**Im**)H_2O	(17c)	−183.7	36.4	−147.3	162.9	−24.4
1 + $Cu(H_2O)_3^+$ → $\mathbf{2^I}(H_2O)$ + $2H_2O$ + H^+	(18)	869.3	−40.1	829.2	−783.9	5.3
Im + $Cu(H_2O)_3^+$ → Cu(**Im**)$(H_2O)^+$ + $2H_2O$	(19)	−73.2	−16.6	−89.8	73.5	−16.3
Im + Cu(**Im**)$(H_2O)^+$ → $Cu(\mathbf{Im})_2^+$ + H_2O	(20a)	−120.1	8.5	−111.6	84.2	−27.4
5 + Cu(**Im**)$(H_2O)^+$ → Cu(**Im**)$(H_{13}H_{14})^+$ + H_2O	(20b)	−105.1	−5.2	−110.4	67.0	−43.4
5 + Cu(**Im**)$(H_2O)^+$ → Cu(**Im**)$(H_{14}H_{13})^+$ + H_2O	(20c)	−135.9	2.7	−133.2	102.2	−31.1
1 + Cu(**Im**)$(H_2O)^+$ → $\mathbf{2^I}$(**Im**) + H_2O + H^+	(21)	854.4	−8.9	845.5	−808.5	−3.0
$\mathbf{2^I}$(**Im**) + **Im** → $\mathbf{2^I}(\mathbf{Im})_2$	(22)	−71.6	33.6	−38.1	60.8	22.7
1 + $Cu(\mathbf{Im})_2^+$ → $\mathbf{2^I}(\mathbf{Im})_2$ + H^+	(23)	902.9	16.2	919.1	−831.9	47.2
5 + $Cu(H_2O)_3^+$ → $\mathbf{6^I}$ + $3H_2O$	(24)	−176.3	−23.4	−199.7	126.9	−72.8
1 + $\mathbf{6^I}$ → $\mathbf{8a^I}$ + H^+	(25a)	993.0	−7.1	985.9	−881.3	64.6
1 + $\mathbf{6^I}$ → $\mathbf{8b^I}$ + H^+	(25b)	940.3	17.0	957.3	−853.9	63.4
NMA + $\mathbf{6^I}$ → $\mathbf{7^I}$	(26)	−59.2	26.0	−33.2	58.2	25.0
Im + $\mathbf{6^I}$ → $\mathbf{6^I}$(**Im**)	(27)	−74.2	27.9	−46.3	56.4	10.1
2a$(H_2O)_2$ + e^- → $\mathbf{2a^I}(H_2O)$ + H_2O	(28)	−641.7	−42.0	−683.7	215.9	−467.8[b]
2a(**Im**)(H_2O) + e^- → $\mathbf{2^I}$(**Im**) + H_2O	(29)	−624.7	−31.4	−656.1	187.8	−468.3[b]
2a$(\mathbf{Im})_2$ + **5** + e^- + H^+ → **1** + Cu(**Im**)$(H_{13}H_{14})^+$	(30a)	−74.2	27.9	−46.3	56.4	10.1[b]
2a$(\mathbf{Im})_2$ + **5** + e^- + H^+ → **1** + Cu(**Im**)$(H_{14}H_{13})^+$	(30b)	−1529.6	−25.9	−1555.4	1030.0	−525.4[b]
2a$(\mathbf{Im})_2$ + **5** + e^- + H^+ → **1** + $\mathbf{6^I}$(**Im**) + **Im**	(31)	−1570.9	−7.4	−1578.3	1037.7	−540.6[b]
2a$(\mathbf{Im})_2$ + **5** + e^- + H^+ → **1** + $\mathbf{6^I}$ + 2**Im**	(32)	−1496.7	−35.3	−1532.1	981.3	−550.8[b]
6$(H_2O)_2$ + e^- → $\mathbf{6^I}$ + $2H_2O$	(33)	−906.1	−64.5	−970.6	442.7	−527.9[b]
6(**Im**)(H_2O) + e^- → $\mathbf{6^I}$ + **Im** + H_2O	(34)	−784.2	−74.1	−858.4	343.0	−515.4[b]
7 + e^- → $\mathbf{6^I}$ + **Im** + NMA	(35)	−715.7	−77.8	−793.5	257.6	−535.9[b]
6(**Im**)(H_2O) + e^- → $\mathbf{6^I}$(**Im**) + H_2O	(36)	−858.4	−46.2	−904.6	399.4	−505.3[b]

[a] Numbered structures are presented in Figures 1–4. [b] The enthalpy correction 57 kJ mol^{-1} has not been added.

The amino and carboxylate groups form adjacent corners of the square, and the carbonyl of the amide occupies the axial position. Two water molecules complete the square base. Structure **2b**$(H_2O)_2$, which is lacking the axial carbonyl oxygen coordination, is less stable than **2a**$(H_2O)_2$ by 32 kJ mol^{-1}. The primary reason for the lower stability of **2b**$(H_2O)_2$ is enthalpic. The Cu–O bond strength of the coordinated carbonyl oxygen of Asp1 is about 50 kJ mol^{-1} (Table 1). The most stable structure with a deprotonated amide N coordinated to the Cu(II) is **2c**$(H_2O)_2$, which is 47 kJ mol^{-1} less stable than **2a**$(H_2O)_2$. The missing proton is on the carboxylate group. Its pK_a is predicted to be 13. In **2c**$(H_2O)_2$, the amide N and the N-terminal NH_2 groups occupy adjacent corners of the square pyramidal base with two waters in the other two corners. The neutral carboxylic acid group of **2c** is attached to the axial position with a long bond (2.43 Å, compared to the average Cu-OH_2 distance, 2.08 Å) [43].

*4.1. Interaction of Cu^{2+}(aq) with the N-Terminal Asp1Ala2 **3**.* The amide-deprotonated structure, **2c**$(H_2O)_2$, is not predicted to be stable in water. However, such a structure permits additional chelation by the carbonyl of the same residue, Ala2 in the present model system. Thus, **3** (i.e., Asp1Ala2) (Figure 2) may provide three ligands for Cu^{2+}(aq) if the amide group is deprotonated. The bicyclic structure, **4**(H_2O) (Figure 2), is the most stable structure that has coordination by the N-terminal NH_2 of Asp1 and the deprotonated amide N and carbonyl O of Ala2 to the Cu^{2+}. All three groups occupy sites in the equatorial coordination plane, the last site being occupied by a water molecule. A second water H bonds to the first and the carboxylic acid group rather than to the copper. The carboxylate group of Asp1 is protonated and interacting with the copper only through H-bonding to the bound water. Equation (6) examines the stability of **4**(H_2O) relative to the dissociated species:

$$\mathbf{3} + Cu(H_2O)_5 \longrightarrow \mathbf{4}(H_2O) + 4H_2O + H^+; \quad \Delta G_{(6)} = 10.2\,\text{kJ mol}^{-1}, \tag{6}$$

where $\Delta G_{(6)}$ has been adjusted to pH = 7. Thus, compared to (5c), coordination by the O of Ala2 provides an additional 19 kJ mol^{-1} of stabilization but is not enough to render the complex stable in water at physiological pH. The predicted pK_a of the carboxylate proton of **4**(H_2O) is 10, indicating that the deprotonated form of **4**(H_2O) *would* be stable at pH = 9.

*4.2. Interaction of Cu^{2+}/Asp1 Complex, **2a**$(H_2O)_2$, with Imidazole (**Im**).* The side chain of His6 is modeled by imidazole (**Im**). Interaction of Cu^{2+}(aq) with one and two His is represented by:

$$\mathbf{Im} + Cu(H_2O)_5{}^{2+} \longrightarrow Cu(\mathbf{Im})(H_2O)_4{}^{2+} + H_2O; \quad \Delta G_{(7a)} = -0.5\,\text{kJ mol}^{-1}, \tag{7a}$$

$$2\mathbf{Im} + Cu(H_2O)_5{}^{2+} \longrightarrow Cu(\mathbf{Im})_2(H_2O)_3{}^{2+} + 2H_2O; \quad \Delta G_{(7b)} = -10.9\,\text{kJ mol}^{-1}. \tag{7b}$$

The structures of the two **Im**/Cu(II) complexes are shown in Figure 1. Displacement of a water ligand by **Im** is predicted to be isoergonic, $\Delta G_{(7b)} = -0.5$ kJ mol^{-1}. This result derives from the near cancellation of a highly exergonic change in the gaseous phase by a correspondingly large loss of free energy of solvation (the $\Delta G_{(g)}$ and $\Delta\Delta G_{solv}$ terms in Table 1). It is in disagreement with results derived from early pH measurements which found $\log_{10}\beta_1 = 3.76$ or $\Delta G_1 = -21$ kJ mol^{-1} [44]. Addition of the second **Im** was found experimentally to have $\log_{10}\beta_2 = 3.39$ or $\Delta G_1 = -19$ kJ mol^{-1} [44]. The displacement of a second water is calculated to be more favored, by 10 kJ mol^{-1}. The computed results suggest that a monoadduct will disproportionate to form the bisadduct and aqueous Cu(II). The preferred orientation of the imidazole rings in Cu(**Im**)$_2(H_2O)_3$ is perpendicular to the basal square plane. In the bis(**Im**) complex, both *cis*- and *trans*-diastereomers are stable and are nearly isoergonic. In the *cis*-structure, only one of the imidazole rings is almost perpendicular to the basal plane. The single **Im** in Cu(**Im**) $(H_2O)_4{}^{2+}$ is intended to represent His6 or one of His13 or His14. The second **Im** of Cu(**Im**)$_2(H_2O)_3{}^{2+}$ may be one of His13 or His14, or a His6 from a second Aβ. Thus, the 1 : 1 interaction of Cu^{2+}(aq) with **Im** is less favorable than with Asp1 by 18 kJ mol^{-1} and the 1 : 2 interaction is comparable.

The most stable structures, **2a**(**Im**)(H_2O) and **2a**(**Im**)$_2$, for the 1 : 1 : 1 and 1 : 1 : 2 complexes, respectively, between Cu^{2+}(aq), Asp1, and **Im** are shown in Figure 1. Structure **2a**(**Im**)(H_2O) is related to **2a**$(H_2O)_2$ by the substitution of the water that is anti to the carboxylate group by **Im**. The basal plane with the axially coordinated amide carbonyl is preserved. Substitution in **2a**$(H_2O)_2$ of the water anti to the amino group yielding **2b**(**Im**)(H_2O) results in loss of the axial ligand and distortion of the basal plane. Structure **2a**(**Im**)(H_2O) is more stable than **2b**(**Im**)(H_2O) by 38 kJ mol^{-1} (from data in Table S2).

Substitution of both waters of **2a**$(H_2O)_2$ by **Im** yields **2a**(**Im**)$_2$. A second structure, **2b**(**Im**)$_2$, in which the **Im** residues are opposite the NH_2 and amide carbonyl groups, and the carboxylate group occupies the axial position, is also stable. In **2b**(**Im**)$_2$, the carboxylate group is coordinated through one of the oxygen atoms but perpendicular to the CO_2 plane. Structure **2a**(**Im**)$_2$ is more stable than **2b**(**Im**)$_2$ (Figure 1) by 11 kJ mol^{-1}. Equations (8a) and (8b) explore the possible reactions that may yield the 1 : 1 : 1 Cu^{2+}(aq), Asp1, and **Im** adduct, **2a**(**Im**)(H_2O):

$$\mathbf{2a}(H_2O)_2 + \mathbf{Im} \longrightarrow \mathbf{2a}(\mathbf{Im})(H_2O) + H_2O; \quad \Delta G_{(8a)} = -24.1\,\text{kJ mol}^{-1}, \tag{8a}$$

$$\mathbf{1} + Cu(\mathbf{Im})(H_2O)_4{}^{2+} \longrightarrow \mathbf{2a}(\mathbf{Im})(H_2O) + 3H_2O + H^+; \quad \Delta G_{(8b)} = -41.6\,\text{kJ mol}^{-1}, \tag{8b}$$

where $\Delta G_{(8b)}$ has been adjusted to pH = 7. Thus, **2a**(**Im**)(H_2O) is stable with respect to dissociation either by releasing an imidazole ligand or the N-terminal Asp. In the context to Aβ, these results imply that Asp1 **1** and any of the His residues may form a stable complex with Cu(II) in water at physiological pH.

The possible formation of the 1 : 1 : 2 complex, **2a**(**Im**)$_2$, is explored in reactions (9a) and (9b):

$$\mathbf{2a}(\mathbf{Im})(H_2O) + \mathbf{Im} \longrightarrow \mathbf{2a}(\mathbf{Im})_2 + H_2O; \quad \Delta G_{(9a)} = -11.0\,\mathrm{kJ\,mol^{-1}}, \tag{9a}$$

$$\mathbf{1} + \mathrm{Cu}(\mathbf{Im})_2(H_2O)_3{}^{2+} \longrightarrow \mathbf{2a}(\mathbf{Im})_2 + 3H_2O + H^+; \quad \Delta G_{(9b)} = -42.2\,\mathrm{kJ\,mol^{-1}}, \tag{9b}$$

where $\Delta G_{(9b)}$ has been adjusted to pH = 7. Thus, as with **2a**(**Im**)(H_2O), the 1 : 1 : 2 complex, **2a**(**Im**)$_2$, is also stable toward dissociation, and, in the context to Aβ, these results imply that Asp1, His6, and either of His13 or His14 may form stable complexes in water at physiological pH.

By (6), it was apparent that deprotonation of the amide N (of Ala2) required coordination of the O of Ala2 in order to afford a complex, **4**(H_2O), that could be formed at pH = 9 but was not stable at pH = 7. Equations (10a) and (10b) examine the possibility that the water of **4**(H_2O) may be displaced by **Im**:

$$\mathbf{4}(H_2O) + \mathbf{Im} \longrightarrow \mathbf{4}(\mathbf{Im}) + H_2O; \quad \Delta G_{(10a)} = -20.5\,\mathrm{kJ\,mol^{-1}}, \tag{10a}$$

$$\mathbf{3} + \mathrm{Cu}(H_2O)_5{}^{2+} + \mathbf{Im} \longrightarrow \mathbf{4}(\mathbf{Im}) + 5H_2O + H^+; \quad \Delta G_{(10b)} = -10.3\,\mathrm{kJ\,mol^{-1}}, \tag{10b}$$

where $\Delta G_{(10b)}$ has been adjusted to pH = 7. Thus, the additional stabilization afforded by replacing water by **Im** (10a), is sufficient to render the product, **4**(**Im**) (Figure 2), stable in water at physiological pH (10b), but would have only marginal stability at lower pH, $\Delta G_{(10b)} = -4\,\mathrm{kJ\,mol^{-1}}$ at pH = 6. Structure **4**(**Im**) has a tetracoordinate square planar configuration. The carboxylate group of Asp1 is H-bonded to the NH_2 group and not interacting with the copper. Deprotonation of **4**(**Im**) affords **4**(**Im**)($CO_2{}^-$) (Figure 2) with pK_a = 11. **4**(**Im**)($CO_2{}^-$) is pentacoordinated with the carboxylate group occupying the fifth site. In the context of Aβ, (10a) and (10b) imply that a 3N1O complex incorporating the N-terminal NH_2 group, the carbonyl O, and the deprotonated amide N of Ala2, and one of His6, His13 or H14, should be observed at physiological pH:

$$\mathbf{4}(\mathbf{Im}) + \mathbf{Im} \longrightarrow \mathbf{4}(\mathbf{Im})_2; \quad \Delta G_{(11)} = 6.9\,\mathrm{kJ\,mol^{-1}}. \tag{11}$$

By (11), coordination of a second **Im** to the vacant axial coordination site of **4**(**Im**) to yield **4**(**Im**)$_2$ (Figure 2) is unfavorable by 7 kJ mol^{-1}. Such a structure may be an intermediate for the interchange of the His residues of Aβ.

But is a deprotonated amide structure like **4**(**Im**) stable compared to a form like **2a**(**Im**)$_2$ in which the amide is protonated and Ala2 is not involved? Equation (12) compares the stability of the most stable copper-coordinated structure that uses both Asp1 and Ala2, **4**(**Im**), with one that does not involve Ala2, namely, **2a**(**Im**)$_2$:

$$\mathbf{4}(\mathbf{Im}) + \mathbf{Im} + \mathbf{1} \longrightarrow \mathbf{2a}(\mathbf{Im})_2 + \mathbf{3}; \quad \Delta G_{(12)} = -42.8\,\mathrm{kJ\,mol^{-1}}. \tag{12}$$

The relatively large exergonicity of (12), $\Delta G_{(12)} = -43\,\mathrm{kJ\,mol^{-1}}$, strongly suggests that Ala2 is *not* involved in the bonding in the N-terminal copper-bound species. The principal reason, from Table 1, is enthalpic ($\Delta H_{(12)} = -84\,\mathrm{kJ\,mol^{-1}}$ and is a consequence of the greater acidity of the carboxylate group than of the amide group. Thus, **2a**(**Im**)$_2$ is the closest model for Component I of Aβ, but raising the pH, at least in the physiological range, does not lead to Component II as proposed by Faller and Hureau and coworkers [6, 7]. In the following section, we propose another structure for Component II and discuss the nature of the pH dependence.

4.3. Interaction of Cu^{2+} with His13His14 ***5***. We explore below the special case of His13His14 where the **Im** groups of the two His residues are tethered by an intervening amide link. The tethering has an important consequence. As with **3**, this configuration permits three-point chelation to the copper, a favorable contribution to the free energy of binding, but without the penalty of amide deprotonation.

The His13His14 sequence of Aβ is modeled by **5** (Figure 3), in which only the two side chains and the intervening amide link are preserved [17]. The interaction between **5** and Cu^{2+}(aq) yielding **6**(H_2O)$_2$ was recently studied experimentally and reexamined theoretically by the procedures employed in the present paper [18]. The calculated association constant, $\log_{10}K_{(12)}$ for (13), was in good agreement with the experimental value, $\log_{10}K_{as} = 5.6$ [18]:

$$\mathbf{5} + \mathrm{Cu}(H_2O)_5{}^{2+} \longrightarrow \mathbf{6}(H_2O)_2 + 3H_2O; \quad \Delta G_{(13)} = -36.0\,\mathrm{kJ\,mol^{-1}}; \quad \log_{10}K_{(13)} = 6.3. \tag{13}$$

Structure **6**(H_2O)$_2$, which models the mode of attachment of Cu(II) to His13His14, has the two **Im** groups in the *trans*-positions of a distorted square plane. The backbone amide carbonyl oxygen and a water molecule occupy the other two opposing sites. A second water occupies the apical site of the square pyramid. The *trans*-orientation of the two **Im** groups is the favored mode of attachment as seen in *trans*-Cu(**Im**)$_2$(H_2O)$_3$ (Figure 1). It has been argued that the copper does not attach to a HisHis sequence through the N_δ atoms in a *trans*-arrangement as in **6**(H_2O)$_2$, but rather through the N_ε atoms in a *cis*-arrangement [1]. The latter is the configuration seen in the crystal structure of bis(cyclo-L-histidyl-L-histidyl)copper(II), the cyclic anhydride of histidine [45]. In this compound, Cu^{2+} chelates to the N_ε atom of the imidazole rings in a *cis*-arrangement. In the

present system, the most stable structure in which Cu(II) is attached to **5** with the cis coordination pattern, is $\mathbf{6}^{\varepsilon\varepsilon}(H_2O)_3$ (Tables S1 and S2)). Structure $\mathbf{6}^{\varepsilon\varepsilon}(H_2O)_3$ is predicted to be less stable relative to $\mathbf{6}(H_2O)_2 + H_2O$ by 36 kJ mol^{-1}. The constraint imposed by the framework of the cyclic anhydride of histidine does not permit bridging of the copper ion through one or both N_δ atoms of the **Im** groups. In **5**, or in monomeric Aβ, there are no such constraints. We also found structures, $\mathbf{6}^{\delta\varepsilon}(H_2O)_2$ and $\mathbf{6}^{\varepsilon\delta}(H_2O)_2$ (Tables S1 and S2)), in which coordination is through one N_δ and one N_ε nitrogen of **5**. These also have the cis orientation and are even less stable, 46 and 51 kJ mol^{-1} (data in Table S2). We do not consider these structures to be relevant to the chemistry of Cu/Aβ in Alzheimer's disease and do not discuss them further.

Species $\mathbf{6}(H_2O)_2$ may add an additional **Im** residue yielding, $\mathbf{6}(\mathbf{Im})(H_2O)$. The results are presented in:

$$\mathbf{6}(H_2O)_2 + \mathbf{Im} \longrightarrow \mathbf{6}(\mathbf{Im})(H_2O) + H_2O\ ; \quad \Delta G_{(14)} = -12.5\,\text{kJ mol}^{-1}. \tag{14}$$

The remaining water is in an axial orientation with a long Cu-O separation, 2.40 Å. Exchange of the water by an O of an amide carbonyl group (of NMA = N-methylacetamide) yields **7** (Figure 3). In **7**, the two carbonyl groups occupy equivalent positions in the equatorial plane of a trigonal bipyramidal configuration about the Cu(II) ion. Attempts to optimize square planar structures with the O of His13 or NMA in an axial position converged to similar trigonal bipyramidal structures. The reaction is described in:

$$\mathbf{6}(\mathbf{Im})(H_2O) + \text{NMA} \longrightarrow \mathbf{7} + H_2O; \quad \Delta G_{(15)} = -20.5\,\text{kJ mol}^{-1}; \quad \log_{10}K_{(15)} = 3.6. \tag{15}$$

Reaction (15) is moderately exergonic in water, indicating that the Cu(II) environment could consist of the three His residues and an additional carbonyl group. In the context of Cu/Aβ, the experimental results of Barnham and coworkers [12], and the theoretical modeling of Sodupe and coworkers [14], on Cu(II)/Aβ(1-16), the obvious candidate for the additional carbonyl O is Ala2. We note however that displacement of water by NMA is strongly *endothermic* in the gaseous phase, $\Delta H_{(g)} = 68\,\text{kJ mol}^{-1}$ (Table 1). This is a consequence of the steric crowding about the Cu(II) site that forces the unusual trigonal bipyramidal geometry. The exergonicity of reaction (15) in water ensues from an even larger favorable change in the free energy of solvation, $\Delta\Delta G_{(aq)} = -84\,\text{kJ mol}^{-1}$ (Table 1). Solvation was also found to favor the coordination of the O of Ala2 in the work of Sodupe and coworkers [14].

*4.4. Interaction of Cu^{2+}/Asp1Complex, **2a**(H_2O), with His13His14 **5**.* The most stable structures corresponding to the 1 : 1 : 1 complex between Cu^{2+}(aq), Asp1, and His13His14 (**5**), namely, **8a** and **8b**, are shown in Figure 2. The more stable of the two, **8a**, has a square pyramidal coordination sphere about the copper, with bidentate coordination of the $-NH_2$ and $-CO_2^-$ groups of the Asp in the basal square. One of the **Im** groups (formally of His13) and the backbone amide carbonyl group of **5** form the other two corners. The other **Im** group (formally of His14) has moved into the apical site. The second structure, **8b**, which is less stable by 18 kJ mol^{-1}, has the same square pyramidal 3N1O basal configuration as **8a,** but the apical **Im** group has released. Possible routes for the formation of the more stable isomer **8a** are examined in:

$$\mathbf{1} + \mathbf{6}(H_2O)_2 \longrightarrow 8\mathbf{a} + 2H_2O + H^+; \quad \Delta G_{(16a)} = +2.8\,\text{kJ mol}^{-1}, \tag{16a}$$

$$\mathbf{2a}(H_2O)_2 + \mathbf{5} \longrightarrow \mathbf{8a} + 2H_2O; \quad \Delta G_{(16b)} = -15.2\,\text{kJ mol}^{-1}, \tag{16b}$$

where $\Delta G_{(16a)}$ has been adjusted to pH = 7. The negative free energy change of (16b), $\Delta G_{(16b)} = -15$ kJ mol^{-1}, indicates that if the cupric ion was already attached to $\mathbf{2a}(H_2O)_2$, that is, the N-terminus, it can also associate with **5**, that is, His13His14. However, the small positive value, $\Delta G_{(16a)} = +3$ kJ mol^{-1}, indicates that **8a** would be partially dissociated, releasing the N-terminal Asp. Equation (17a) addresses the question of whether the N-terminus can be coordinated if there are already three His residues coordinated to the copper ion. Such a structure, **9**, is shown in Figure 3:

$$\mathbf{1} + \mathbf{6}(\mathbf{Im})(H_2O) \longrightarrow \mathbf{9} + H_2O + H^+; \quad \Delta G_{(17a)} = +22.0\,\text{kJ mol}^{-1}, \tag{17a}$$

$$\mathbf{9} \longrightarrow \mathbf{8a} + \mathbf{Im}; \quad \Delta G_{(17b)} = -6.7\,\text{kJ mol}^{-1}, \tag{17b}$$

$$\mathbf{9} + H_2O + H^+ \longrightarrow \mathbf{5} + \mathbf{2a}(\mathbf{Im})(H_2O); \quad \Delta G_{(17c)} = 24.4\,\text{kJ mol}^{-1}, \tag{17c}$$

where $\Delta G_{(17a)}$ and $\Delta G_{(17c)}$ have been adjusted to pH = 7. The moderately high value, $\Delta G_{(17a)} = 22\,\text{kJ mol}^{-1}$, indicates that simultaneous attachment of all four potential ligands to Cu(II), the N-terminal Asp and the three histidines, is not likely at physiological pH. Equations (17b) and (17c) indicate that such an arrangement would be unstable with respect to loss of one histidine but not two.

In the context of Aβ, the overall picture that emerges from the above considerations is that the N-terminus and all three His residues may not be simultaneously associated with the cupric ion. However, such a structure could be a transitional form connecting more stable structures in which the Cu^{2+}(aq) is attached to His6, His13, and His14 ($\mathbf{6}(\mathbf{Im})(H_2O)$ or **7**) (Figure 3) or to the N-terminus and *two* of the three His residues, His6 and His13, or His6 and His14 (both modeled by $\mathbf{2a}(\mathbf{Im})_2$) (Figure 1) but not His13 and His14 (**8a** is unstable by (16a)) (Figure 3). Thus, structure $\mathbf{2a}(\mathbf{Im})_2$, with 3N1O equatorial coordination to Cu(II), serves as a model for Components Ia and Ib. In each case the equatorial O ligand is one of the carboxylate O atoms of Asp1. Structures $\mathbf{6}(\mathbf{Im})(H_2O)$ or **7** serve as

models for Component II. **6**(**Im**)(H_2O) has the observed 3N1O coordination pattern. Structure **7** is preferred because it is more stable, but it formally has a 3N2O coordination pattern in a trigonal bipyramid. The computed results for Cu(II) species agree in most respects with experimental expectations, except possibly for the nature of the O ligand, which would be the carbonyl O atom of His13 if **6**(**Im**)(H_2O) proves to be the better model for Component II. However, favoring **7** in this respect also is the experimental [12] and other computational [14] evidence that the "O" should be the O atom of Ala2 provided the approximately equivalent equatorial O atoms of the trigonal bipyramidal geometry would manifest as 3N1O coordination in EPR experiments. Attempts on our part to completely displace the O of His13 in Cu(II) complexes by any other ligand always failed.

If Component I is modeled by **2a**($\mathbf{Im}$)$_2$ and Component II is modeled by **7**, what then is the nature of the pH dependence that shifts the equilibrium from one to the other in the narrow physiological pH range? We suggest that, since the pK_a of His residues is in this range, one or more of the His residues would be protonated. The presence of the Cu^{2+} ion sets up a delicate balance: in Component I, either His13 or His14 is protonated, permitting the other to bind to the copper together with His6 and Asp1 (but not Ala2); at a slightly higher pH, the remaining His is deprotonated and all three can bind to the copper, displacing Asp1 but leaving the nearby O of Ala2 attached.

5. Interaction of Cu^+(aq) with the N-Terminal Asp1 **1**

The most stable form of the aqueous cuprous ion at the present theoretical level is the T-shaped triaqua structure, $Cu(H_2O)_3^+$ (Figure 4), Reaction with Asp1 **1** yielded several aquated structures with different patterns of chelation. The most stable of these is the 1 : 1 Cu(I) : Asp1 complex, $\mathbf{2^I}(H_2O)$ (Figure 4, see the following equation):

$$\mathbf{1} + Cu(H_2O)_3{}^+ \longrightarrow \mathbf{2^I}(H_2O) + 2H_2O + H^+; \quad \Delta G_{(18)} = +5.3\,\text{kJ mol}^{-1}, \tag{18}$$

where $\Delta G_{(18)}$ has been adjusted to pH = 7. We denote structures containing the cuprous ion by the superscript **I** on the structure number of the Cu(II) equivalent. As with $Cu(H_2O)_5{}^{2+}$ and $Cu(H_2O)_3{}^+$, the cuprous form of a complex will generally have fewer ligands than the equivalent cupric form. Some or all of the attached H_2O will be lost upon reduction of the copper. If there is no attached water, then one or more of the coordinated ligands will be released upon reduction. The structures of all complexes containing Cu(I) are shown in Figure 4.

The small endergonic free energy change for reaction (18) in water, $\Delta G_{(18)} = 5\,\text{kJ mol}^{-1}$ at pH = 7, suggests that there is a small amount of 1 : 1 complex formed between Cu^+(aq) and Asp1 under physiological conditions. We examine whether complexation of Asp1 is feasible if the cuprous ion is already attached to one or more imidazoles. The reactions for the 1 : 1 complex between Cu^+(aq) and **Im**, Cu(**Im**)$(H_2O)^+$, is given in:

$$\mathbf{Im} + Cu(H_2O)_3{}^+ \longrightarrow Cu(\mathbf{Im})(H_2O)^+ + 2H_2O; \quad \Delta G_{(19)} = -16.3\,\text{kJ mol}^{-1}. \tag{19}$$

Thus, Cu(**Im**)$(H_2O)^+$ is stable in water and the free energy of complexation is higher than for the equivalent Cu(II) complex (7a). The following reactions explore the addition of a second imidazole group to Cu(**Im**)$(H_2O)^+$, either as free **Im** or as His13 or His14 of **5**, yielding products Cu(**Im**)$_2{}^+$ (20a), Cu(**Im**)$(H_{13}H_{14})^+$ (20b), and Cu(**Im**)$(H_{14}H_{13})^+$ (20c), respectively:

$$\mathbf{Im} + Cu(\mathbf{Im})(H_2O)^+ \longrightarrow Cu(\mathbf{Im})_2{}^+ + H_2O; \quad \Delta G_{(20a)} = -27.4\,\text{kJ mol}^{-1}, \tag{20a}$$

$$\mathbf{5} + Cu(\mathbf{Im})(H_2O)^+ \longrightarrow Cu(\mathbf{Im})(H_{13}H_{14})^+ + H_2O; \quad \Delta G_{(20b)} = -43.4\,\text{kJ mol}^{-1}, \tag{20b}$$

$$\mathbf{5} + Cu(\mathbf{Im})(H_2O)^+ \longrightarrow Cu(\mathbf{Im})(H_{14}H_{13})^+ + H_2O; \quad \Delta G_{(20c)} = -31.1\,\text{kJ mol}^{-1}. \tag{20c}$$

Thus, addition of His14 of **5** is approximately equivalent energetically to the addition of a free **Im** group and both are more exergonic than addition of a single **Im** (19). Addition of His13 (20b) is more exergonic still due to a higher free energy of solvation (Table 1) which ensues as a consequence of the higher dipole moment of Cu(**Im**)$(H_{13}H_{14})^+$, μ = 15.4 D compared to μ = 13.2 D for Cu(**Im**)$(H_{14}H_{13})^+$.

Both 1 : 1 : 1 and 1 : 1 : 2 complexes between Cu(I), Asp1 **1**, and **Im**, namely, $\mathbf{2^I}$(**Im**) and $\mathbf{2^I}(\mathbf{Im})_2$ (Figure 4), are stable in the gaseous phase. Formation of the 1 : 1 : 1 complex, $\mathbf{2^I}$(**Im**) (Figure 4), is essentially isoergonic at pH = 7 if one **Im** is already attached to Cu(I):

$$\mathbf{1} + Cu(\mathbf{Im})(H_2O)^+ \longrightarrow \mathbf{2^I}(\mathbf{Im}) + H_2O + H^+; \quad \Delta G_{(21)} = -3.0\,\text{kJ mol}^{-1}, \tag{21}$$

where $\Delta G_{(21)}$ has been adjusted to pH = 7. Addition of a second **Im** to form the 1 : 1 : 2 complex, $\mathbf{2^I}(\mathbf{Im})_2$ (Figure 4), is endergonic, $\Delta G_{(22)} = 23\,\text{kJ mol}^{-1}$:

$$\mathbf{2^I}(\mathbf{Im}) + \mathbf{Im} \longrightarrow \mathbf{2^I}(\mathbf{Im})_2; \quad \Delta G_{(22)} = 22.7\,\text{kJ mol}^{-1}. \tag{22}$$

Because of the high affinity of Cu(I) for two imidazoles, addition of Asp1 **1** to the bis(imidazole)Cu(I) complex will not occur:

$$\mathbf{1} + Cu(\mathbf{Im})_2^+ \longrightarrow \mathbf{2^I}(\mathbf{Im})_2 + H^+; \quad \Delta G_{(23)} = 47.2\,\text{kJ mol}^{-1}, \tag{23}$$

where $\Delta G_{(23)}$ has been adjusted to pH = 7. On the basis of this result, we have not attempted to add Asp1 to Cu(**Im**)$(H_{13}H_{14})^+$ or Cu(**Im**)$(H_{14}H_{12})^+$.

*5.1. Interaction of Cu^+ and/Asp1 **1**, with His13His14 **5**.* Cu(I) binds to the HisHis region **5** yielding the 1 : 1 complex, $\mathbf{6^I}$ (Figure 4), with high affinity,

$$\mathbf{5} + Cu(H_2O)_3{}^+ \longrightarrow \mathbf{6^I} + 3H_2O;$$
$$\Delta G_{(24)} = -72.8\,\text{kJ mol}^{-1}; \quad \log_{10}K_{(24)} = 12.7. \tag{24}$$

Coordination of additional waters to $\mathbf{6^I}$ is endergonic, for example, $\Delta G \geq 15\,\text{kJ mol}^{-1}$ for the addition of one water (data not shown). The calculated affinity constant, $\log_{10}K_{(23)} = 12.7$, is in very good agreement with that found for the Cu(I)/Aβ(1-16) complex, $\log_{10}K_{\text{aff}} \approx 14$ [24]. The value is substantially higher than for the addition of two free imidazoles to Cu^+(aq) (20a), or a free **Im** and one of the two imidazoles of **5** ((20b) and (20c)), thus highlighting the importance of chelation. It is also higher than for the addition of Cu^{2+}(aq) to HisHis (13). The calculations clearly confirm [30] that Cu(I) will bind more strongly than Cu(II) to Aβ and that the preferred site of binding of Cu(I) is His13His14. The linear geometry and a Cu-N distance of 1.877 Å in Cu(I)/Aβ(1-40) was deduced from fitted EXAFS data by Shearer and Szalai [23]. Our calculated value for $\mathbf{6^I}$, 1.894 Å, is in good agreement.

Addition of Asp1 **1** to the linear Cu(I)/HisHis complex, $\mathbf{6^I}$, in the gaseous phase yields two stable structures, $\mathbf{8a^I}$ and $\mathbf{8b^I}$ (Figure 4), of equal energy in water. However, the formation of either by addition of Asp1 **1** to $\mathbf{6^I}$ in water is highly endergonic ((25a) and (25b)) due to the high stability of $\mathbf{6^I}$:

$$\mathbf{1} + \mathbf{6^I} \longrightarrow \mathbf{8a^I} + H^+; \quad \Delta G_{(25a)} = 64.6\,\text{kJ mol}^{-1}, \tag{25a}$$

$$\mathbf{1} + \mathbf{6^I} \longrightarrow \mathbf{8b^I} + H^+; \quad \Delta G_{(25b)} = 63.4\,\text{kJ mol}^{-1}, \tag{25b}$$

where $\Delta G_{(25a)}$ and $\Delta G_{(25b)}$ have been adjusted to pH = 7. Equations (16a), (16b), and (17a) indicate that the N-terminus will be weakly associated with Cu(II) complexes of two of the His residues. However, upon reduction of the copper to Cu(I), the N-terminus will be released leaving only the linear Cu(I)/HisHis structure. Equation (15) indicates that an amide carbonyl will be weakly associated with Cu(II) complexes of all three His residues. Equation (26) examines whether the carbonyl would remain attached to the reduced copper species, $\mathbf{6^I}$:

$$\text{NMA} + \mathbf{6^I} \longrightarrow \mathbf{7^I};$$
$$\Delta G_{(26)} = 25.0\,\text{kJ mol}^{-1}. \tag{26}$$

The complex with NMA (N-methylacetamide), $\mathbf{7^I}$ (Figure 3), is strongly bound in the gaseous phase, $\Delta H_{((26),\text{g})} = -59\,\text{kJ mol}^{-1}$, but is formed endergonically in water due to a combination of loss of solvation free energy and an unfavorable entropic term (Table 1). Addition of **Im** to $\mathbf{6^I}$ to yield $\mathbf{6^I}$(**Im**) (Figure 4) is slightly endergonic,

$$\mathbf{Im} + \mathbf{6^I} \longrightarrow \mathbf{6^I}(\mathbf{Im});$$
$$\Delta G_{(27)} = 10.1\,\text{kJ mol} - 1; \quad \log_{10}K_{(27)} = -1.8. \tag{27}$$

As with NMA, the addition reaction is endergonic principally by virtue of loss of free energy of solvation, but also in part due to the unfavorable change in entropy (Table 1).

In the context of a reduced copper/Aβ complex, there is ample experimental evidence that Cu(I) is bound to His13His14 precisely as depicted in $\mathbf{6^I}$, in a linear fashion through the N_δ of both **Im** groups (Figure 4) [21, 22, 46]. All other ligands, including the third **Im**, are released upon reduction. The **Im** (His6) is tethered to the Cu(I) binding region at His13His14, and the loss of entropy may be less, reducing the endergonicity of (27). There is experimental evidence that a third **Im** can associate transiently with Cu(I)/Aβ [26].

6. Reduction Potentials for Cu(II) Complexes with the N-Terminal Asp1 1

Computed reduction potentials for various combinations of Cu(II)/Aβ and Cu(I)/Aβ coordination patterns, when compared with the experimental value for Cu/Aβ, $E^\circ \approx 0.30\,\text{V}$–$0.34\,\text{V}$, [28, 29, 47] may serve to distinguish among the different possibilities that have been suggested in the literature and discussed in the Introduction. Since all experimental indications suggest that Aβ and Cu/Aβ are highly fluxional species, the most stable structures should be most populated. We assume that electrochemical reduction is an equilibrium process. Logically then, reduction of the (predicted) most stable Cu(II) species, yielding the most stable Cu(I) species, should yield the most representative value of E° or $E^{\circ\prime}$. As a second point of reference, the experimental value for the reduction of aqueous cupric ion is $E^\circ(Cu^{2+}(\text{aq})/Cu^+(\text{aq})) = 0.17\,\text{V}$ [41]. We now examine possible redox scenarios in the Cu/Aβ context.

Equation (28) describes the reduction process if the copper was attached only to the N-terminal Asp1:

$$\mathbf{2a}(H_2O)_2 + e^- \longrightarrow \mathbf{2a^I}(H_2O) + H_2O;$$
$$E^\circ\left(\mathbf{2a}(H_2O)_2/\mathbf{2a^I}(H_2O)\right) = -0.07\,\text{V}. \tag{28}$$

The calculated standard reduction potential $E^\circ(\mathbf{2a}(H_2O)_2/\mathbf{2a^I}(H_2O)) = -0.07\,\text{V}$, is lower than the value for aqueous copper. Therefore, Cu(II) cannot be attached only to the N-terminus since the lower E° is incompatible with the experimental observation that the reduction potential is elevated. A lower value is expected since a negatively charged group is attached to the Cu(II), thereby lowering the net charge of the oxidized species.

Equation (29) describes reduction of copper attached in a 1 : 1 ratio to Asp1 and **Im** The Cu(II) species, $\mathbf{2a}(\mathbf{Im})(H_2O)$, was found to be stable in water ((8a) and (8b)), but the Cu(I) species, $\mathbf{2^I}(\mathbf{Im})$, had only transient stability (21):

$$\mathbf{2a}(\mathbf{Im})(H_2O) + e^- \longrightarrow \mathbf{2^I}(\mathbf{Im}) + H_2O;$$
$$E^\circ\left(\mathbf{2a}(\mathbf{Im})(H_2O)/\mathbf{2^I}(\mathbf{Im})\right) = -0.07\,\text{V}. \tag{29}$$

The result, $E^\circ(\mathbf{2a}(\mathbf{Im})(H_2O)/\mathbf{2^I}(\mathbf{Im})) = -0.07\,\text{V}$, indicates that the presence of the **Im** moiety has little effect on the predicted reduction potential.

With respect to the possibility that Cu(II) may be coordinated to Asp1 and two of the **Im** moieties, the Cu(II) species, **2a**$(\mathbf{Im})_2$, was found to be stable at pH = 7 ((9a) and (9b)). Structure **2a**$(\mathbf{Im})_2$ corresponds to Component I, the low pH species, in which Cu(II) is attached to His6 and either His13 or His14 as well as the N-terminus. The corresponding reduced species, $\mathbf{2^I}(\mathbf{Im})_2$ was found to release the Asp (23). Equations (30a) and (30b) describe the appropriate reduction process if the Cu(I) of Component I remains attached to the same two His residues:

$$\mathbf{2a}(\mathbf{Im})_2 + \mathbf{5} + e^- + H^+ \longrightarrow \mathbf{1} + \mathrm{Cu}(\mathbf{Im})(H_{13}H_{14})^+;$$
$$E^{\circ\prime}\left(\mathbf{2a}(\mathbf{Im})_2, H^+/\mathrm{Cu}(\mathbf{Im})(H_{13}H_{14})^+, \mathbf{1}\right) = 0.24\,\mathrm{V}, \tag{30a}$$

$$\mathbf{2a}(\mathbf{Im})_2 + \mathbf{5} + e^- + H^+ \longrightarrow \mathbf{1} + \mathrm{Cu}(\mathbf{Im})(H_{14}H_{13})^+;$$
$$E^{\circ\prime}\left(\mathbf{2a}(\mathbf{Im})_2, H^+/\mathrm{Cu}(\mathbf{Im})(H_{14}H_{13})^+, \mathbf{1}\right) = 0.11\,\mathrm{V}. \tag{30b}$$

If, during the reduction process, the most stable Cu(I) product, $\mathbf{6^I}$, is formed, the process may be modeled by (31) in which His6 is retained or by (32) in which His6 is released:

$$\mathbf{2a}(\mathbf{Im})_2 + \mathbf{5} + e^- + H^+ \longrightarrow \mathbf{1} + \mathbf{6^I}(\mathbf{Im}) + \mathbf{Im};$$
$$E^{\circ\prime}\left(\mathbf{2a}(\mathbf{Im})_2, \mathbf{5}, H^+/\mathbf{6^I}(\mathbf{Im}), \mathbf{1}, \mathbf{Im}\right) = 0.27\,\mathrm{V}, \tag{31}$$

$$\mathbf{2a}(\mathbf{Im})_2 + \mathbf{5} + e^- + H^+ \longrightarrow \mathbf{1} + \mathbf{6^I} + 2\mathbf{Im};$$
$$E^{\circ\prime}\left(\mathbf{2a}(\mathbf{Im})_2, \mathbf{5}, H^+/\mathbf{6^I}, \mathbf{1}, \mathbf{Im}\right) = 0.37\,\mathrm{V}. \tag{32}$$

In summary, the reduction potentials of Cu(II) attached to the N-terminus and one or two independent His residues ((30a) and (30b)) in which the Cu(I) remains attached to the same two His residues are predicted to be slightly lower than the experimental value for Cu(II)/Aβ complexes, 0.30 V–0.34 V. However, if the Cu(I) rearranges to include both His13 and His14, with or without loss of His6 ((31) and (32), resp.), E° values are predicted to be close to the experimental value. Therefore, it is possible that Component I is the species that is observed to undergo reduction.

Component II, modeled by **6**$(\mathbf{Im})(H_2O)$ or **7**, does not involve the N-terminal Asp1. Reduction of either species in which Cu(II) is coordinated to His13His4 will yield the Cu(I)/HisHis species, $\mathbf{6^I}$, with the release of all other coordinating ligands. In the instance that Cu(II) is *only* attached to HisHis except for waters (33), an elevated value of E° is obtained:

$$\mathbf{6}(H_2O)_2 + e^- \longrightarrow \mathbf{6^I} + 2H_2O;$$
$$E^\circ\left(\mathbf{6}(H_2O)_2/\mathbf{6^I}\right) = 0.55\,\mathrm{V}. \tag{33}$$

The high result confirms that **6**$(H_2O)_2$ is also an incomplete description of the bonding in Cu(II)/Aβ. A more representative species is **6**$(\mathbf{Im})(H_2O)$, which models all three His residues coordinated to Aβ with a coordinated water:

$$\mathbf{6}(\mathbf{Im})(H_2O) + e^- \longrightarrow \mathbf{6^I} + \mathbf{Im} + H_2O;$$
$$E^\circ\left(\mathbf{6}(\mathbf{Im})(H_2O)/\mathbf{6^I}, \mathbf{Im}\right) = 0.42\,\mathrm{V}. \tag{34}$$

The E° value (34) is close to but higher than the experimental range. The species, **6**$(\mathbf{Im})(H_2O)$, has a coordinated water molecule which may be exchanged for another ligand. On the basis of the experimental finding that the O atom of Ala2 is in the Cu(II) coordination sphere of Component II, we consider here structure **7**, in which the water is replaced by an amide carbonyl, using N-methylacetamide (NMA) as a model. The substrate, **7,** is shown in Figure 3. The reduction of **7** is described in:

$$\mathbf{7} + e^- \longrightarrow \mathbf{6^I} + \mathbf{Im} + \mathrm{NMA};$$
$$E^\circ\left(\mathbf{7}/\mathbf{6^I}, \mathbf{Im}, \mathrm{NMA}\right) = 0.63\,\mathrm{V}. \tag{35}$$

The E° value (35) is substantially higher than the experimental range.

Thus, it appears that none of mechanisms that involve reduction and *spontaneous* loss of ligands provides a satisfactory description of the reduction process. Balland et al., have recently carried out a detailed study of the kinetics of the reduction/oxidation of Cu/Aβ(1-16) complexes by cyclic voltametry and homogeneous transfer from osmium complexes [47]. The electron transfer rate was found to be extremely slow and required a preorganization by 22 and 16 kJ mol^{-1} to geometries of the Cu(II) and Cu(I) species, respectively, between which the actual electron transfer takes place. In the present model systems, the preorganization of the oxidized species is to release the bound carbonyl oxygen, that is, the reverse of (15) for which $\Delta G_{(15)} = 20\,\mathrm{kJ\,mol^{-1}}$. The preorganization of the reduced species prior to oxidation is to gain the **Im** residue, that is, (27) for which $\Delta G_{(27)} = 10\,\mathrm{kJ\,mol^{-1}}$. Thus, in the Balland et al. scheme, the actual reduction is described by:

$$\mathbf{6}(\mathbf{Im})(H_2O) + e^- \longrightarrow \mathbf{6^I}(\mathbf{Im}) + H_2O;$$
$$E^\circ\left(\mathbf{6}(\mathbf{Im})(H_2O)/\mathbf{6^I}(\mathbf{Im}), H_2O\right) = 0.31\,\mathrm{V}. \tag{36}$$

The calculated reduction potential, $E^\circ(\mathbf{6}(\mathbf{Im})(H_2O)/\mathbf{6^I}(\mathbf{Im}), H_2O) = 0.31$ V, is in excellent agreement with that measured by Balland et al., $E^\circ = 0.30$ V, and the calculated free energy changes for the preorganization steps are also in good agreement.

7. Conclusions

High-level ab initio electronic structure calculations were applied to models of the N-terminus of Aβ, as well as Ala2, His6, and His13His14, to predict structures of the complexes of Cu(II) and Cu(I) in water at physiological pH. The calculated binding affinities of both Cu(II) and Cu(I) to the His13His14 model, $\log_{10}K_{\mathrm{aff}} = 6.3$ (13) and $\log_{10}K_{\mathrm{aff}} = 12.7$ (24), respectively, are in good agreement with experimental values, 5.6 [18] and 14 [24], respectively which lends confidence to other calculated free energy changes.

At the present level of theory, Cu(II) species are predicted to be pentacoordinated in a square pyramidal configuration. The one exception we found is in the case of **7** (Figure 3), the proposed model for Component II of Aβ. In **7**, two carbonyl oxygen atoms, of Ala2 and His13, occupy nearly equivalent

sites in the equatorial plane of a trigonal bipyramid. On the other hand, the predominant configuration at Cu(I) in water is linear dicoordination, with the exception of $Cu(H_2O)_3^+$ and the possible exception of Cu(I) with all three His residues attached (modeled by $\mathbf{6^I}(\mathbf{Im})$ (Figure 4)) which are T-shaped structures.

It was found that Asp1 forms stable complexes with Cu(II) and *two* His residues, either the pair His6, His13, or the pair His6, His14, but not His13His14, both modeled by $\mathbf{2a}(\mathbf{Im})_2$ (Figure 1). Complexes involving the deprotonated amide of Ala2 are substantially less stable. The complex, $\mathbf{2a}(\mathbf{Im})_2$, represents the bonding configuration of Cu(II) in Component Ia and Component Ib of Aβ. Each has 3N1O square planar coordination with tridentate attachment of Asp1. The $-NH_2$ and $-CO_2^-$ groups contribute one of the N ligands and the O ligand in the equatorial plane, while the carbonyl O of Asp1 occupies the axial position.

The N-terminus does not attach to Cu(II) if His6 and His13His14 are already attached. Structure **7** is the best candidate for Component II. The assignment of **7** as a model for Component II is in agreement with the findings of Barnham and coworkers [12]. Hureau and Faller and coworkers suggested that the amide NH of Ala2 is deprotonated at higher pH and proposed a structure for Component II that is modeled by $\mathbf{4}(\mathbf{Im})$ (Figure 2) in our study [6, 7]. Our results suggest that the presence of the carboxylate group of Asp1 makes deprotonation of the amide group very improbable near physiological pH. Rather, we propose that deprotonation of a protonated His residue of either His13 or His14 facilitates the formation of Component II at the expense of Component I.

The binding configuration of Cu(I) to Aβ is modeled by $\mathbf{6^I}$ (Figure 4). The Cu(I) is linearly dicoordinated to His13His14 through the N_δ nitrogen atoms of the imidazole groups. Weak coordination of His6, as in $\mathbf{6^I}(\mathbf{Im})$, is possible in water as an endergonic process.

Our calculations support in full the redox scheme for Cu/Aβ(1-16) proposed by Balland et al., which requires preorganization steps for both oxidized and reduced species [47]. The sequence of steps for reduction is described by the reverse of (15) and (27), and (36), which we repeat here for clarity:

$$\mathbf{7} + H_2O \longrightarrow \mathbf{6}(\mathbf{Im})(H_2O) + \text{NMA};$$
$$\Delta G_{(15')} = 20\,\text{kJ mol}^{-1};\ \left[22\,\text{kJ mol}^{-1}\right], \qquad ((15'))$$

$$\mathbf{6}(\mathbf{Im})(H_2O) + e^- \longrightarrow \mathbf{6^I}(\mathbf{Im}) + H_2O;$$
$$E^\circ(\mathbf{6}(\mathbf{Im})(H_2O)/\mathbf{6I}(\mathbf{Im}), H_2O) = 0.31\,\text{V};\ [0.30\,\text{V}], \qquad ((36'))$$

$$\mathbf{6^I}(\mathbf{Im}) \longrightarrow \mathbf{6^I} + \mathbf{Im};$$
$$\Delta G_{(27')} = -10\,\text{kJ mol}^{-1};\ \left[-16\,\text{kJ mol}^{-1}\right]. \qquad ((27'))$$

The experimental values derived from the data of Balland et al. are given in square parentheses. The reoxidation occurs by the exact reverse sequence.

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Research Article

The Cellular Prion Protein Prevents Copper-Induced Inhibition of $P2X_4$ Receptors

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Although the physiological function of the cellular prion protein (PrP^C) remains unknown, several evidences support the notion of its role in copper homeostasis. PrP^C binds Cu^{2+} through a domain composed by four to five repeats of eight amino acids. Previously, we have shown that the perfusion of this domain prevents and reverses the inhibition by Cu^{2+} of the adenosine triphosphate (ATP)-evoked currents in the $P2X_4$ receptor subtype, highlighting a modulatory role for PrP^C in synaptic transmission through regulation of Cu^{2+} levels. Here, we study the effect of full-length PrP^C in Cu^{2+} inhibition of $P2X_4$ receptor when both are coexpressed. PrP^C expression does not significantly change the ATP concentration-response curve in oocytes expressing $P2X_4$ receptors. However, the presence of PrP^C reduces the inhibition by Cu^{2+} of the ATP-elicited currents in these oocytes, confirming our previous observations with the Cu^{2+} binding domain. Thus, our observations suggest a role for PrP^C in modulating synaptic activity through binding of extracellular Cu^{2+}.

1. Introduction

Prion diseases are a group of fatal neurodegenerative disorders that are sporadic, inherited, or transmissible [1]. These include kuru and Creutzfeldt-Jakob disease in humans, scrapie in sheep and bovine spongiform encephalopathy in cattle. These pathologies are caused by the conformational transition of the native and predominantly α-helical cellular prion protein (PrP^C) into a significantly more β-sheet-containing pathogenic isoform (PrP^{Sc}) [2], which unlike PrP^C, is insoluble in mild detergents and partially resistant to digestion with proteinase K [3]. PrP^C is a cell surface glycosylphosphatidylinositol-anchored protein that is mainly expressed in neurons and glial cells and to a lesser extent in several peripheral tissues [4, 5]. The normal physiological function of PrP^C remains elusive, although it has been related to signaling, neuroprotection, neuritogenesis, synaptic transmission, oxidative stress, and copper metabolism [6–11].

PrP^C binds copper ions with low micromolar affinity via histidine and glycine-containing peptide repeats in its N-terminal region [12–17]. This Cu^{2+} binding domain is located between residues 60–91 and consists of four identical repeats of the peptide sequence Pro-His-Gly-Gly-Gly-Trp-Gly-Gln. Although the number of octapeptide repeats varies in different species, in mammals this region is one of the most highly conserved [18] and therefore, very likely defines a functional domain of PrP^C. *In vitro*, the octarepeat region has the capacity to reduce Cu(II) to Cu(I) [19, 20]. In addition, there is another Cu^{2+} binding site outside the octarepeat region [21–24] of higher affinity, in the order of nanomolar, that involves His96 and His111 [24]. PrP^C is localized presynaptically at central synapses [25–27] and is found in synaptic membranes and in synaptic vesicles [9, 28]. Furthermore, PrP^C-null mice show an impaired long-term potentiation, suggesting that PrP^C is involved in normal synaptic function [10], and moreover, it has been shown

that PrP^C is involved in regulating the presynaptic Cu^{2+} concentration and synaptic transmission [9].

The P2X family of nucleotide receptors forms nonselective cationic channels activated by extracellular adenosine triphosphate (ATP) [29]. These receptors are widely expressed in the central nervous system (CNS) [30–32] and are involved in synaptic transmission and plasticity including long-term potentiation as recently shown by us [33]. Interestingly, trace metals modulate P2X receptors, particularly, the $P2X_4$ receptor subtype is differentially modulated by trace metals at physiological concentrations [34–37]. While Zn^{2+} facilitates the ATP-evoked currents, Cu^{2+} inhibits it in a concentration-dependent manner [37]. Previously, we demonstrated that the N-terminal octarepeat fragment of the PrP^C prevents and reverses the inhibitory action of Cu^{2+} on the $P2X_4$ receptor when added to the media [38]. Herein, in an attempt to determine whether the PrP^C-Cu^{2+} interaction is relevant to synaptic activity, we extended our investigations to test whether the full-length PrP^C coexpressed with the $P2X_4$ receptor may modulate *in situ* the Cu^{2+}-induced inhibition of the ATP current gated by the $P2X_4$ receptor.

2. Materials and Methods

2.1. Drugs and Chemicals. Copper chloride, ATP (as the tetrasodium salt), collagenase IA, and penicillin-streptomycin were purchased from Sigma Chemical Co (St Louis, Mo). All the salts used to prepare the Barth's incubation media and the recording solutions were analytically graded and were purchased from Merck (Darmstadt, Germany).

2.2. Oocyte Preparation, Injection, and Electrophysiological Recordings. A segment of the *Xenopus laevis* ovary lobe was surgically removed from adult anesthetized frogs; stages V-VI oocytes were manually defolliculated and then incubated with collagenase IA (1 mg/mL) for 30 min. Oocytes were manually injected with 7.5–12.5 ng cDNA coding for the rat $P2X_4$ receptor with or without cDNA coding for the hamster prion protein (PrP-3F4), both cDNAs in plasmid pcDNA3, at 250 ng/μL. After 48–72 h of incubation at 15°C in Barth's solution (in mM): 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 10 HEPES, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, pH 7.5, supplemented with 10 IU/L penicillin/10 mg streptomycin, oocytes were clamped at −70 mV using the two-electrode voltage clamp technique with an OC-725C oocyte clamper (Warner Instrument Corp, Hamden, CT). ATP and $CuCl_2$, dissolved in Barth's solution, were superfused at 2 ml/min. ATP-evoked currents were recorded with a 10 s ATP exposure applied regularly at 10–15 min intervals. These intervals were increased up to 25 min for maximal ATP concentrations in concentration-response curves protocols to decrease desensitization. Copper was applied for 30 s prior 10 μM ATP (coapplied with $CuCl_2$).

2.3. Confocal Microscopy. To study the distribution of PrP, oocytes were coinjected with the cDNA coding for the rat $P2X_4$ receptor with the cDNA coding for mouse PrP-GFP (MmPrP-EGFP[25-266]-cDNA3). Oocytes, where $P2X_4$ receptor expression was validated electrophysiologically, were directly analyzed on a Zeiss LSM 5 Pascal confocal microscope.

2.4. Western Blotting. After electrophysiological protocols, each oocyte injected with cDNa coding for $P2X_4$ and PrP-3F4 was homogenized for 30 min in ice, using 40 μL of lysis buffer per oocyte (100 mM NaCl, 20 mM Tris-HCl pH 7.4, 1% Triton X-100) supplemented with a protease inhibitors cocktail [39]. The extracts were centrifuged for 30 s at 14000 r.p.m. at 4°C and the supernatant was removed and resolved by 12% SDS-PAGE and transferred to nitrocellulose. Nonspecific binding sites were blocked with 5% (w/v) milk in Tris-Buffered Saline (TBS) 0.1% Tween (TBST) for 1 h. After blocking, blots were incubated with monoclonal anti-3F4 antibody [40], diluted 1 : 5000 in 3% (w/v) milk in TBST for 1 h at room temperature, followed by three 15 min washes in TBST at room temperature. The reactions were followed by incubation with anti-mouse antibody peroxidase labeled (Pierce, Rockford, IL) and developed by enhanced chemiluminescence.

2.5. Data Analysis. The average reduction of the ATP-gated current was normalized. The ATP and Cu^{2+} concentration-response curves were fitted to a sigmoid function using the GraphPad Prism software (San Diego, Cal). The median effective (EC_{50}) or median inhibitory concentrations (IC_{50}) for ATP or copper, respectively, were interpolated from these curves. Each protocol was performed in separate oocytes coming from at least two separate batches of oocytes. Mann-Whitney nonparametric Student's t-test was used for statistical analysis. A P value < 0.05 was considered significant.

3. Results

3.1. The Expression of PrP-3F4 Did Not Change the ATP Concentration-Response Curve of $P2X_4$ Receptors. To evaluate whether the expression of PrP^C modulates the inhibition of the $P2X_4$ receptor by Cu^{2+}, we first evaluated the expression of PrP^C in oocytes co-injected with the cDNA coding for the hamster prion protein (PrP-3F4) and the cDNA coding for the rat $P2X_4$ receptor. Figure 1(a) shows the detection by western blot of $P2X_4$ receptor and PrP-3F4 using an antibody that recognizes the 3F4 epitope [40]. β-Tubulin detection was used as a loading control. As observed, both proteins are strongly detected in an injected oocyte and not in the control noninjected oocyte. Then we analyzed the distribution of PrP^C in oocytes co-injected with the cDNA coding for the rat $P2X_4$ receptor and the cDNA coding for PrP-GFP. Oocytes in which the expression of $P2X_4$ receptor was verified electrophysiologically were analyzed in a confocal microscope to study the localization of PrP-GFP. As observed in Figure 1(b), PrP-GFP is located on the surface of injected oocytes.

Then, we evaluated the ATP concentration-response curves in oocytes expressing the $P2X_4$ receptor and coexpressing the $P2X_4$ receptor and PrP-3F4. The presence of PrP-3F4 caused a slight, but not significant, reduction in

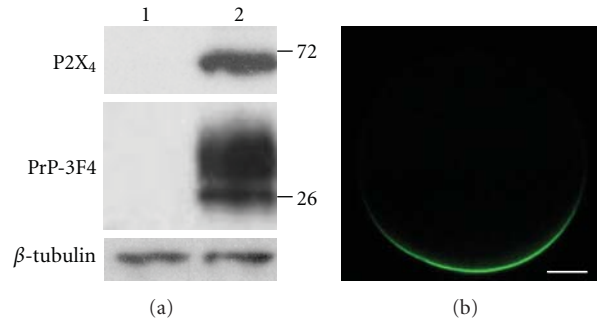

FIGURE 1: Coexpression of $P2X_4$ and PrP^C in *X. laevis* oocytes. (a) Western blot of total lysate fractions from a non-injected oocyte (*left lane*, 1) and from an oocyte co-expressing $P2X_4$ receptor and PrP-3F4 (*right lane*, 2). Numbers on the right are molecular weights in kDa. (b) Fluorescence microscopy of an oocyte co-expressing $P2X_4$ receptor and PrP-GFP (green), bar = 10 μM.

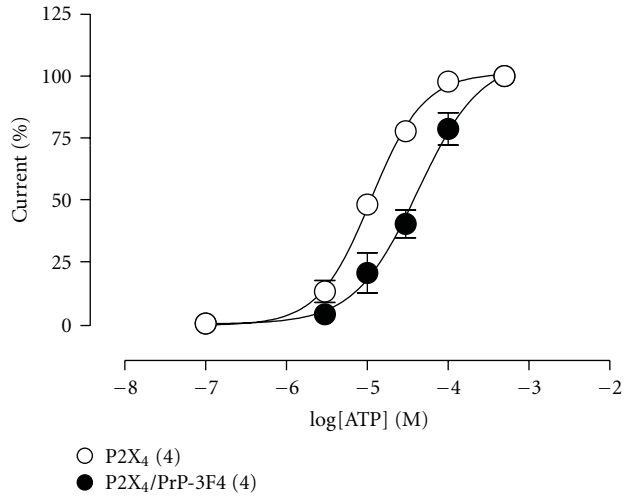

FIGURE 2: ATP concentration-response curves from oocytes expressing $P2X_4$ receptor (open circles) or co-expressing $P2X_4$ receptor and PrP-3F4 (closed circles). Symbols are mean values ± SEM, numbers in parenthesis are number of oocytes.

the potency of ATP, reflected as an increase in its EC_{50} from 11.2 ± 1.1 μM for $P2X_4$ alone to 45.2 ± 9.4 μM for $P2X_4$/PrP-3F4 ($n = 4$, $P = 0.0571$, Figure 2), this slight displacement of ATP concentration-response curve in the presence of PrP-3F4 could represent a minor regulation of PrP-3F4 on $P2X_4$ receptor activity.

3.2. The Co-Expression of $P2X_4$ Receptors and PrP-3F4 Partially Prevents the Copper-Induced Inhibition of the ATP-Evoked Currents. We assess the Cu^{2+}-induced inhibition of 10 μM ATP currents in oocytes expressing $P2X_4$ receptors. The magnitude of the inhibition by 10 μM Cu^{2+}, preapplied during 30 s, was 51.5 ± 5.3% of the 10 μM ATP-evoked currents ($n = 14$, Figures 3(a) and 3(b)). However, the 10 μM Cu^{2+}-induced inhibition was reduced only to 71.9 ± 5% of the 10 μM ATP-evoked currents in oocytes co-expressing $P2X_4$ receptors and the PrP-3F4 ($n = 12$, $P < 0.05$ compared to $P2X_4$ alone, Figures 3(a) and 3(b)), showing that PrP-3F4 prevented the Cu^{2+}-induced inhibition of $P2X_4$ receptors compared to the Cu^{2+} inhibition elicited in oocytes expressing only this receptor. Furthermore, the presence of PrP-3F4 in the oocytes caused a rightward displacement of the Cu^{2+} concentration-response curve obtained in oocytes expressing only $P2X_4$ receptor, an IC_{50} of 11.5 ± 1.9 μM was obtained for $P2X_4$ and 34.1 ± 7.6 μM for $P2X_4$/PrP-3F4 ($n = 5$–7, $P < 0.01$, Figure 3(c)), confirming that PrP-3F4 prevented the Cu^{2+}-induced inhibition not only at low micromolar concentrations of Cu^{2+}, but even at higher physiological concentrations of the metal.

4. Discussion

Several functions have been attributed to PrP^C, including immunoregulation, signal transduction, copper binding, neurite outgrowth, induction of apoptosis or prevention of apoptosis against apoptotic stimuli, and others [41]. In addition, PrP^C has been related to synapse formation and maintenance and synaptic transmission [9, 10, 42], although the mechanisms by which it exerts its role is still unknown. One of the proposed targets for PrP^C in synapse is to modulate Cu^{2+} homeostasis, based on a highly conserved Cu^{2+}-binding sequence located on its N-terminal domain, which includes four identical repeats of the peptide sequence Pro-His-Gly-Gly-Gly-Trp-Gly-Gln [12, 15, 16]. It is known that PrP^C binds Cu^{2+} with high affinity [14–17], and the octarepeat region of the human PrP^C (PrP_{59-91}) reduces Cu(II) to Cu(I) *in vitro*, which depends on the tryptophan residues present in the octapeptide repeats [19, 20]. Cu^{2+} modulates synaptic transmission at micromolar concentrations by a wide range of mechanisms, be one of the most relevanting modulations of neurotransmitter receptors within glutamatergic, gabaergic, and purinergic synapses, among others [43, 44]. In a previous study, we demonstrated that Cu^{2+} at micromolar concentrations inhibits the ATP-evoked currents of $P2X_4$ receptors [37]. Here we show that the full-length prion protein-expressed in *Xenopus* oocytes localizes in the cell surface and modulates the Cu^{2+} interaction with $P2X_4$ receptor; oocytes which coexpressed PrP-3F4 and $P2X_4$ receptors have a diminished Cu^{2+}-induced inhibition of the ATP-evoked currents compared with oocytes which only expressed the $P2X_4$ receptor. This reduced inhibition by Cu^{2+} was observed on Cu^{2+} concentration-response curves, where the IC_{50} of Cu^{2+} was significantly increased in the presence of PrP-3F4, indicating that PrP-3F4 can exert its modulatory role even at high micromolar concentrations of Cu^{2+}, reached in the synaptic cleft after depolarization [45]. These results, together with our previous findings showing that coapplication of Cu^{2+} with the N-terminal PrP fragment (PrP_{59-91}) prevents the inhibitory effect of copper on $P2X_4$ receptors and even reverts the established Cu^{2+}-induced inhibition of the $P2X_4$ receptors [38], strongly support the idea that PrP^C could modulate synaptic copper and therefore affect the function of $P2X_4$ receptors and synaptic transmission.

In addition to the potential synaptic role of PrP^C driven by its ability to bind Cu^{2+}, a known modulator of

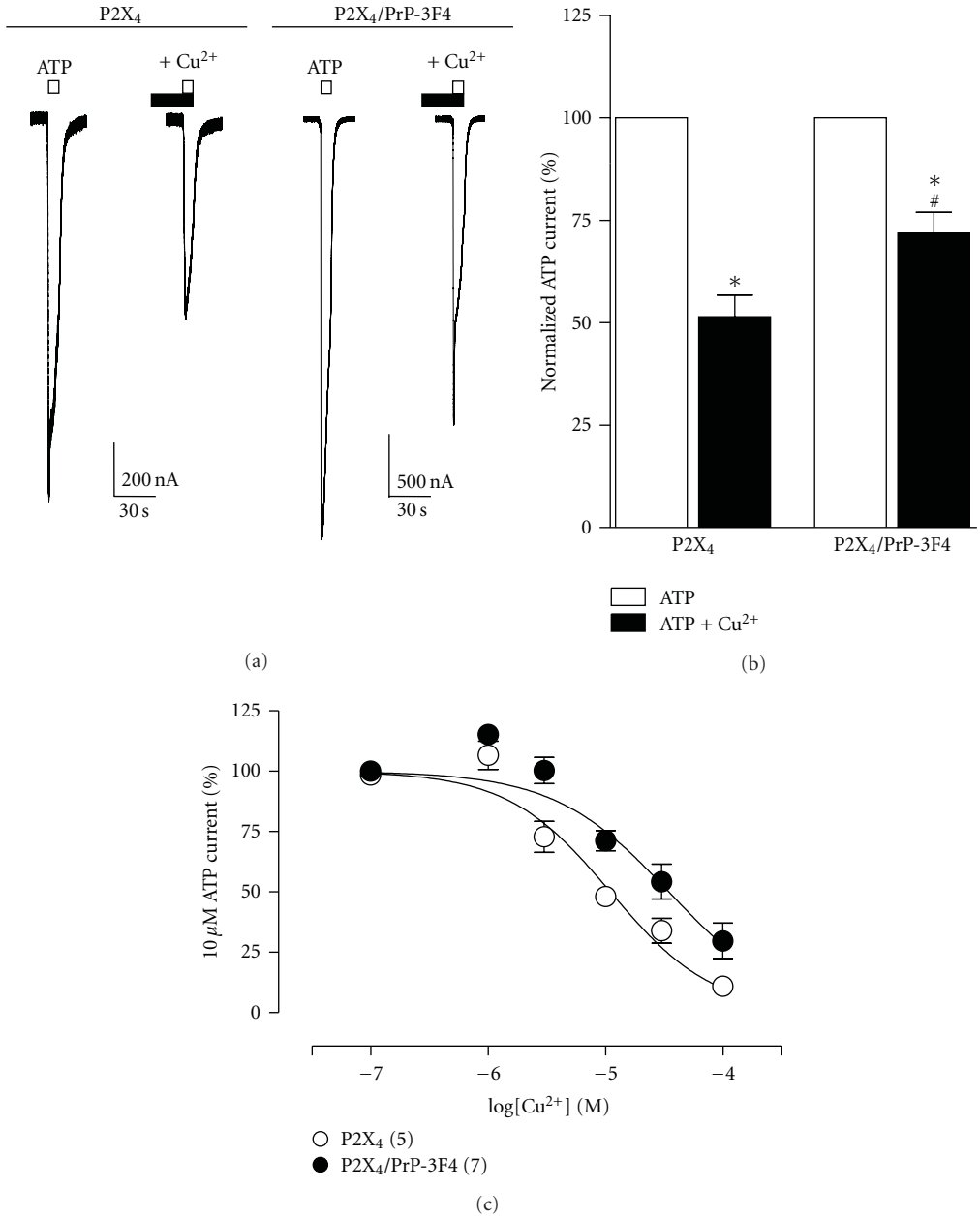

FIGURE 3: PrP^C prevents Cu^{2+}-induced inhibition of $P2X_4$ receptor. (a) Representative recordings obtained from oocytes expressing $P2X_4$ receptor (left traces, $P2X_4$) or coexpressing $P2X_4$ receptor and PrP-3F4 (right traces, $P2X_4$/PrP-3F4) showing 10 μM ATP-evoked currents (*open bars*) and its inhibition by 10 μM Cu^{2+} (*closed bars*). (b) Statistical analysis of Cu^{2+} inhibition showed in (a), performed in different oocytes (n = 12–14, $^*P < 0.01$ versus ATP, $^{\#}P < 0.01$ versus $P2X_4$ alone). Bars are mean values ± SEM. (c), Cu^{2+} concentration-response curves of 10 μM ATP inhibition in oocytes expressing $P2X_4$ receptor (open circles) or co-expressing $P2X_4$ receptor and PrP-3F4 (closed circles). Symbols are mean values ± SEM, numbers in parenthesis are number of oocytes.

neuronal excitability [43, 44], there is increasing evidence of direct interaction between PrP^C and neurotransmitter receptors. PrP^C directly interacts with the NR2D subunit of the NMDA receptor, inhibiting it and preventing NMDA-induced excitoxicity in the hippocampus [46]. On the other hand, PrP^C also exerts a neuroprotective role against kainate-induced neurotoxicity in the hippocampus, probably by regulating differentially the expression of GluR6 and GluR7 kainate receptor subunits [47]. Moreover, PrP^C can modulate the activity of serotoninergic receptors signaling pathways in $1C11^{5-HT}$ cells [48]. We observed a slight, although not significant, reduction on ATP affinity of $P2X_4$ receptor in the presence of PrP-3F4, this might suggest an interference with ATP binding or stabilization of closed states, although further experiments are required to evaluate this hypothesis. Altogether, these studies and the presented here highlight the modulatory role of PrP^C at synaptic transmission in CNS, involving direct regulation of neurotransmitter receptors

and/or their signaling cascade, or indirectly, by controlling the synaptic levels of Cu^{2+}.

The understanding of the physiological function of PrP^C on synaptic transmission may clarify the pathogenic processes underlying prion diseases. Based on our results, it is possible to suggest that the resulting cognitive deterioration of prion diseases could involve a loss of the modulatory role of PrP^C on brain function, as it is converted to the pathogenic isoform.

Abbreviations

PrP^C: Cellular prion protein
ATP: Adenosine triphosphate
CNS: Central nervous system
EC_{50}: Median effective concentration
IC_{50}: Median inhibitory concentration.

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Research Article

Copper Inhibits NMDA Receptor-Independent LTP and Modulates the Paired-Pulse Ratio after LTP in Mouse Hippocampal Slices

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Copper misregulation has been implicated in the pathological processes underlying deterioration of learning and memory in Alzheimer's disease and other neurodegenerative disorders. Supporting this, inhibition of long-term potentiation (LTP) by copper (II) has been well established, but the exact mechanism is poorly characterized. It is thought that an interaction between copper and postsynaptic NMDA receptors is a major part of the mechanism; however, in this study, we found that copper (II) inhibited NMDA receptor-independent LTP in the CA3 region of hippocampal slices. In addition, in the CA3 and CA1 regions, copper modulated the paired-pulse ratio (PPR) in an LTP-dependent manner. Combined, this suggests the involvement of a presynaptic mechanism in the modulation of synaptic plasticity by copper. Inhibition of the copper-dependent changes in the PPR with cyclothiazide suggested that this may involve an interaction with the presynaptic AMPA receptors that regulate neurotransmitter release.

1. Introduction

Copper is a trace element that plays many important roles in the brain, one of the most copper-rich organs of the body. It is an essential structural component and cofactor for many proteins and enzymes, including various effectors of synaptic plasticity, suggesting that it has an important role in regulating the cellular processes underlying learning and memory [1, 2]. The importance of copper in learning and memory is underscored in neurodegenerative diseases including Alzheimer's, Menkes', Wilson's, and Prion disease, in which misregulation of copper is strongly associated with learning and memory deficits [1, 3–5]. It is estimated that copper is present in the extracellular space of brain tissue at a concentration between 0.2 and 1.7 μM; however, it is released during neurotransmission into some glutamatergic synapses where it transiently reaches levels estimated to rise to as high as a few hundred micromolar [6–8]. Synaptically released copper may regulate synaptic plasticity by dampening NMDA, AMPA, and GABA receptor function, as each of these receptors is inhibited by copper at concentrations ranging from the low nanomolar for GABA receptors, to the low micromolar, for AMPA and NMDA receptors [9–14]. However, very little is currently known about the precise mechanisms by which copper interacts with the cellular processes governing learning and memory.

Doreulee et al. (1997) first reported that long-term potentiation (LTP) in the CA1 region of the rodent hippocampus is inhibited by 1 μM copper (II) when present in the extracellular solution bathing slice preparations [15]. LTP, the activity-dependent strengthening of synaptic communication, is a form of synaptic plasticity that is widely accepted as a major mechanism underlying learning and memory [16, 17]. The inhibition of LTP by copper was later repeated and was also observed in brain slices of rats after chronic ingestion or intraperitoneal injection of copper; however, the mechanism behind the inhibition has not been characterized [18–20]. Investigators have suggested that the mechanism may be NMDA receptor dependent; however, it has not yet been investigated whether inhibition of LTP by copper can occur without a contribution from NMDA receptors. It was also shown in one study using slices from the CA1

region of the hippocampus that copper affected the paired-pulse ratio (PPR), a marker of short-term plasticity that is expressed presynaptically [18]. Therefore, in this study, we investigated the inhibition of NMDA receptor-independent LTP by copper in the CA3 region of mouse hippocampal slices. Complete inhibition was observed, and, because LTP in this region is expressed presynaptically, a presynaptic mechanism was suggested. This was further supported by our additional studies showing that the PPR was modulated by copper in both the CA3 and CA1 regions in a manner that was entirely dependent upon the expression of LTP. These results demonstrate that copper can affect presynaptic function during its modulation of hippocampal synaptic plasticity and, therefore, extends our understanding of its mechanism of action beyond a more simple model that involves only the postsynaptic machinery.

2. Materials and Methods

2.1. Brain Slice Preparation. Electrophysiological experiments were conducted in accordance with the Colorado State University-Pueblo Institutional Animal Care and Use Committee (IACUC) guidelines essentially as described previously [21]. Briefly, Swiss Webster mice were housed in standard conditions with 12 hours of light and 12 hours of dark. They were given unlimited access to lab chow and tap water that was filtered using a household pitcher-style filter (Brita). Males and females were used at random between the ages of 1 to 3 months. Mice were sacrificed by decapitation, and a Lancer Vibratome Series 1000 was used to make 350 μM transverse brain slices through the hippocampus. The brain slices were incubated for a minimum of one hour in artificial cerebrospinal fluid (ACSF) supplemented with 2 mM ascorbic acid before being transferred to the recording chamber. ACSF contained (in mM) 124 NaCl, 2.5 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 10 D-glucose, 1.25 NaH_2PO_4, and 26 $NaHCO_3$ and was bubbled vigorously with 95% O_2/5% CO_2. The pH was adjusted with 1 M HCl or 1 M NaOH to between 7.35 and 7.40 and was periodically monitored throughout the course of experimentation. During recording, slices were perfused with oxygenated ACSF at a rate of 3-4 mL per minute at room temperature.

2.2. Stimulation Procedure and Statistics. To measure the field excitatory postsynaptic potential (fEPSP), a 200 μM diameter bipolar concentric stimulating electrode (FHC CBAEC75) and a sharp borosilicate glass recording electrode filled with 2 M NaCl were placed in the stratum radiatum of the CA1 region of the hippocampus at a 250–500 μM interelectrode distance. For CA3 recording, the stimulating electrode was placed on the mossy fiber pathway, and responses were recorded in the stratum lucidum as shown in Figure 1(a). The slice was stimulated with a square pulse of 0.1 ms duration every 30 seconds. To determine the test stimulus intensity, a paired-pulse test was done, and the stimulus intensity was adjusted to the point at which a population spike was evoked on the second, but not the first, pulse as shown in Figure 1(b). This calibration protocol resulted in a stimulus that was 30–50% of the intensity required to elicit a maximum response. The paired-pulse ratio (PPR) was measured at a 50 ms interpulse interval and calculated by dividing the slope of the second fEPSP by the first and converting the ratio to a percentage. For LTP, the baseline fEPSP was obtained for a minimum of 30 minutes, followed by four high-frequency (100 Hz) tetani of one-second duration which were applied in place of the test pulse with a two-minute interval between the second and third tetani. Responses were recorded for 60 minutes after the last tetanus, and the slopes of the fEPSPs were calculated using Clampfit 8 (Axon Instruments). The slopes were averaged over the last five minutes and compared between experimental groups using a paired t-test. For the studies with cyclothiazide (CTZ), a two-sample t-test was used.

2.3. Pharmacological Treatments. Interleaved experiments were conducted with ACSF alone or with 5 μM $CuCl_2$, 5 μM $ZnCl_2$, 100 μM CTZ, 40 μM picrotoxin, and/or 10 μM MK-801. All test solutions were present for the duration of the experiments and were added directly to the ACSF, except for CTZ and picrotoxin, which were dissolved in DMSO; for these experiments, DMSO was also added to the ACSF for the control slices at a final concentration of 0.1%. All chemicals were purchased from Sigma, except for CTZ (A.G. Scientific, Inc.) and MK-801 (Tocris).

3. Results

Extracellular field potential recording was done in the CA1 and CA3 regions of mouse hippocampal slices with and without 5 μM $CuCl_2$ in the recording solution for the duration of experiments. The slopes and waveforms of the fEPSPs, taken after a 30-minute baseline, were not significantly different between control and copper-treated slices in either the CA1 or CA3 regions of the hippocampus (Figures 1(c) and 1(d)). This was consistent with previous reports that 1 μM copper (II) did not affect the slope of the fEPSP but that 10 μM copper (II) depressed it to 85% of control [15]. Thus, 5 μM copper did not appear to affect basal synaptic transmission in our experiments.

Confirming previous studies, 5 μM copper (II) completely blocked LTP of the fEPSP slope in the hippocampal CA1 region of our brain slices (Figures 2(a) and 2(b)) [15, 18, 19]. Also, posttetanic potentiation (PTP) in the CA1 region, measured as the peak fEPSP slope immediately following tetanic stimulation, was somewhat reduced in the presence of copper (Figure 2(c)). LTP in this brain region was dependent on NMDA receptors as it was blocked with DAP5 (data not shown).

To test the requirement for NMDA receptors in the inhibition of LTP by copper, we repeated the above experiments by stimulating the mossy fibers and recording in the CA3 dendritic region with 10 μM MK-801, an NMDA receptor inhibitor, in the bath to isolate NMDA receptor-independent LTP. We were unable to evoke a sufficient LTP to do the experiment in this region; however, with the addition of 40 μM picrotoxin, a GABA receptor inhibitor, robust LTP was present one hour after tetanic stimulation in control slices, and, just as in the CA1 region, copper completely

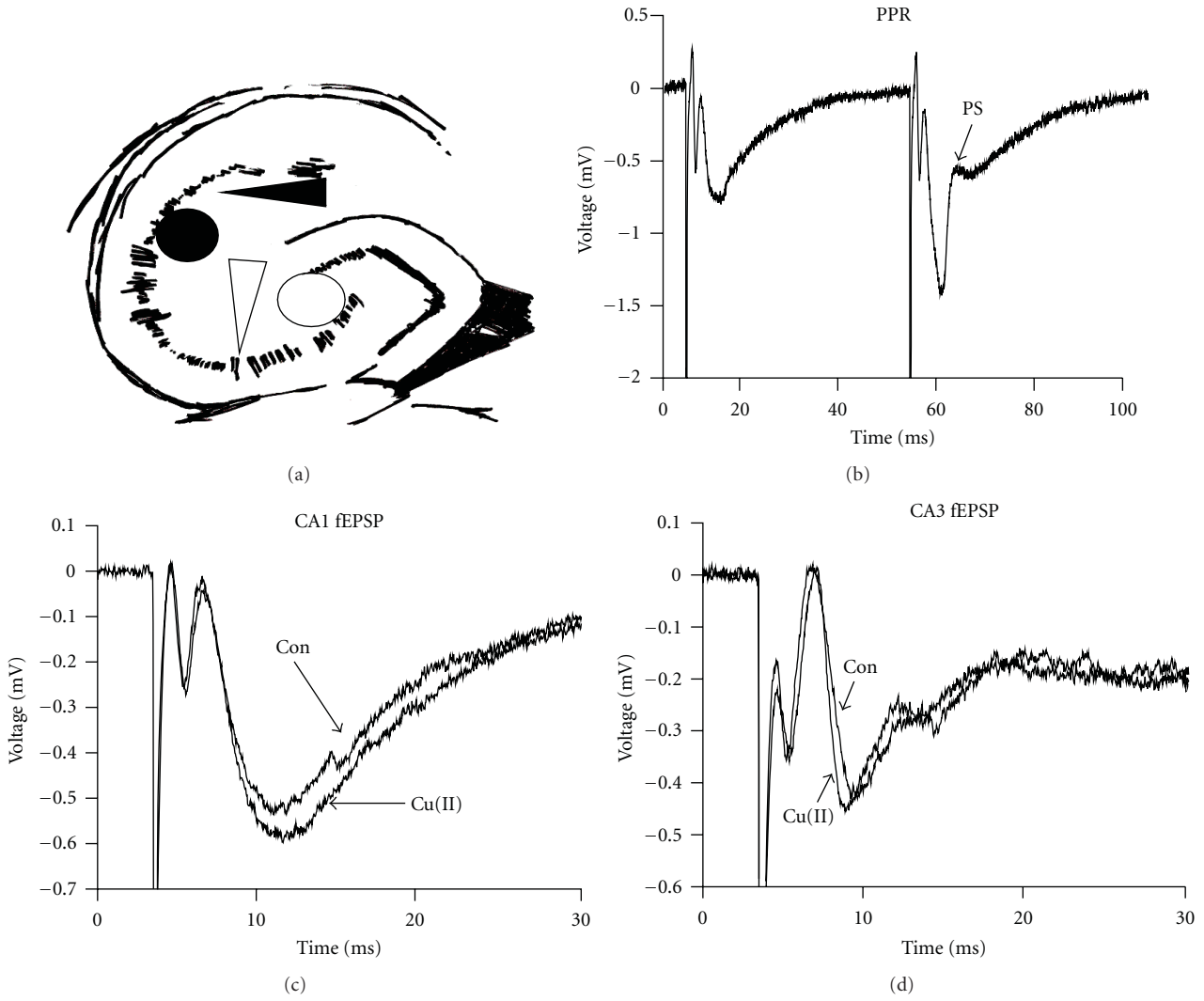

Figure 1: Electrophysiological recording in the mouse hippocampal slice. (a) In the CA1 region, the stimulating electrode (black circle) and the recording electrode (black arrow) were placed in the stratum radiatum. In the CA3 region, the mossy fibers were stimulated (white circle), and recording was done in the stratum lucidum (white arrow). (b) A representative trace from one brain slice showing how a paired-pulse test appeared at the stimulus intensity chosen for recording. (c) and (d) The average of the slopes of the fEPSPs taken 30 seconds before the tetanus was not changed by copper in either the CA1 or CA3 regions ($n = 8$, data not shown). Representative traces from an interleaved experiment are shown.

blocked it (Figures 2(d) and 2(e)). In addition, PTP was statistically significantly reduced by copper in the CA3 region (Figure 2(f)). To the best of our knowledge, these results are the first reported demonstrating that copper inhibits NMDA receptor-independent LTP in the CA3 region of the hippocampus.

Since LTP in the mossy fiber to CA3 cell synapse is thought to be expressed through a presynaptic mechanism [22–25], we were interested in more deeply investigating potential copper-dependent changes in presynaptic plasticity which might occur before and after the induction of LTP. Therefore, we measured the PPR in the presence and absence of 5 μM $CuCl_2$. Confirming previous work, copper did not affect the baseline PPR in the CA1 region (Figure 3(a), left panel), and, in the CA3 region, copper only slightly increased it (Figure 3(a), right panel) [15]. With picrotoxin, which as noted was necessary to achieve LTP in the CA3 region, paired-pulse facilitation was converted to paired-pulse depression (Figure 4). To the best of our knowledge, this lack of an effect of copper on the baseline PPR in the CA3 region has not been reported.

Next, we measured the PPR one hour after inducing LTP in the CA1 and CA3 regions and found that the PPR was significantly enhanced in the presence of copper in the CA1 (Figure 3(a), left panel) but significantly decreased in the CA3 region (Figure 3(a), right panel). To determine whether this effect on the PPR was dependent upon LTP and not simply a result of extended exposure to copper, we incubated the slices for 90 minutes in copper without the LTP-inducing high-frequency stimulus, after which there was no change in

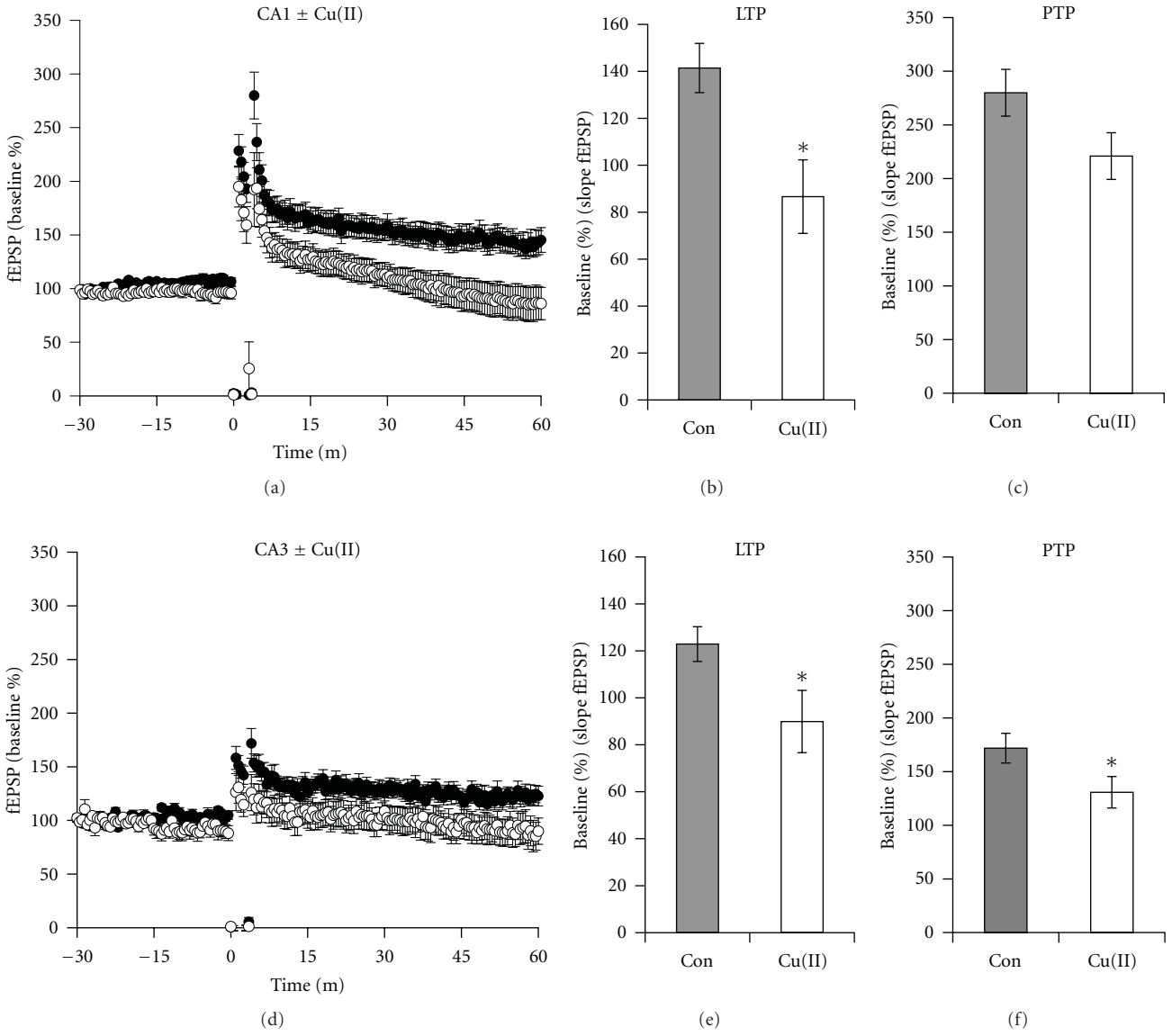

Figure 2: Copper inhibited LTP in the CA1 and CA3 regions of mouse hippocampal slices. (a) In the CA1 region, compared to control slices (black circles), LTP was inhibited by copper (open circles). (b) The average of the fEPSP slopes taken one hour after tetanus was significantly decreased from 141.4 ± 10.5% to 86.6 ± 15.7% in copper-treated slices ($P = 0.034$, $n = 8$). (c) In the CA1 region, the peak PTP was reduced from 280.0 ± 21.8% to 220.9 ± 21.8% in copper-treated slices ($P = 0.069$, $n = 8$). (d) Copper-inhibited LTP in the CA3 region of the mouse hippocampus, control groups are indicated by black circles and copper-treated groups are indicated by open circles. (e) The average fEPSP in the CA3 region measured one hour after tetanus was significantly decreased from 122.9 ± 7.4% to 89.9 ± 13.3% in copper-treated slices ($P = 0.016$, $n = 8$). (f) In the CA3 region, the peak PTP was significantly reduced from 171.8 ± 13.9% to 130.8 ± 14.7% in copper-treated slices ($P = 0.039$, $n = 8$).

the PPR in either hippocampal region (Figure 3(b)). Combined, our results in both regions indicated that LTP caused the appearance of a presynaptic sensitivity to copper which was expressed as a modulation of the PPRs. These results extend the findings of Goldschmith et al. (2005) who showed that rats which had chronically ingested copper displayed changes in the PPR following the expression of LTP in the CA1 region [18].

The effect of copper on PPRs in the CA1 region appeared gradually, as evident when paired-pulse tests were done at intervals preceding and following the tetanus (Figure 5). Over this time course, we found that the enhancement of the PPR matured and became statistically significant only later, at 60 minutes after tetanus, during the maintenance phase of LTP.

While the effects of copper on synaptic plasticity were clearly NMDA receptor independent in the CA3 region, the NMDA receptor dependence is not as easy to ascertain in the CA1 region. Therefore, to more deeply investigate the potential role of NMDA receptors in the CA1 region, we tested

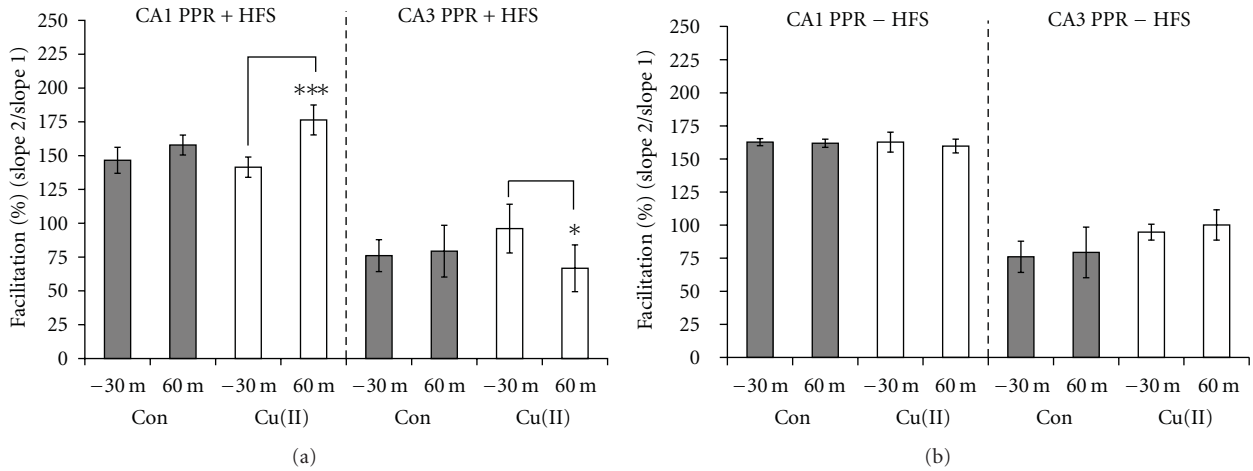

Figure 3: Modulation of the PPR by copper in the CA1 and CA3 regions was dependent on LTP. (a) The average of the PPRs measured 30 minutes before tetanizing the slices was not different in copper-treated (white bars), compared to control slices (gray bars) in either the CA1 or CA3 region. Also, the PPRs in control slices did not change after LTP in either brain region. However, after LTP, copper-treated slices showed a 34.9% enhancement of the PPR in the CA1 region ($P < 0.001$, $n = 8$) and a 29.3% decrease in the PPR in the CA3 region ($P = 0.05$, $n = 8$). (b) Without the HFS, there was no significant change in the PPRs compared between control and copper-treated slices in either the CA1 or CA3 region.

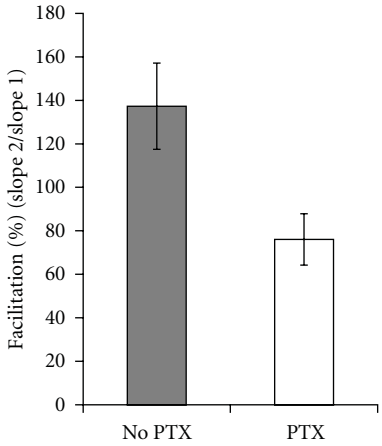

Figure 4: Paired-pulse facilitation was converted to paired-pulse depression by picrotoxin in the CA3 region. The average PPR without picrotoxin was 137.3 ± 19.8% ($n = 4$), but the average PPR with picrotoxin was 76.1% ± 11.8% ($n = 8$).

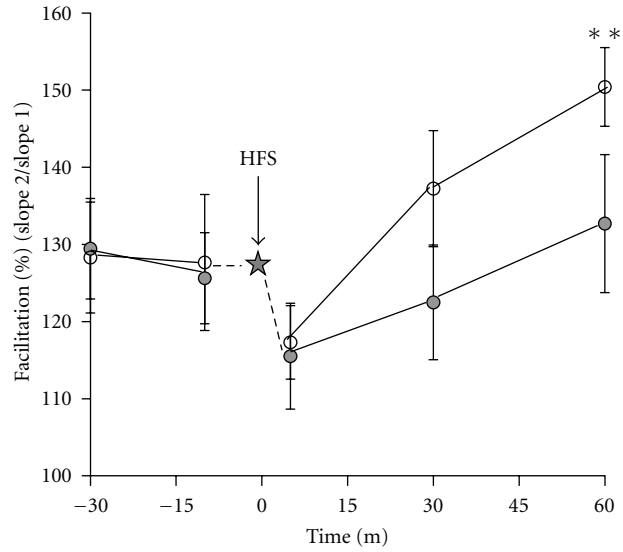

Figure 5: Copper gradually enhanced the PPR after LTP in the CA1 region. Paired-pulse tests were done at intervals before and after the HFS tetanus (star). Prior to the tetanus, the PPR was not significantly different between control slices (filled circles) and copper-treated slices (open circles). Five minutes after the HFS, there was a decrease in the PPR. The difference in the percent facilitation became statistically significant 60 minutes after the tetanus, at which point the average PPR of the copper group was 150.4 ± 5.1% as compared to 132.7 ± 8.9% in the control group ($P = 0.007$, $n = 5$).

whether the zinc modulatory site of the NMDA receptor was a target for copper [26]. Similar to the work of others who have shown that zinc modulates LTP in the CA1 region, LTP was significantly inhibited by 5 μM $ZnCl_2$, just as with copper at an equivalent concentration (Figures 6(a) and 6(b)) [27, 28]. Thus, zinc mimicked the inhibition of LTP by copper. In contrast, zinc and copper had divergent effects on the LTP-dependent changes in the PPR (Figure 6(c)), as there was no significant difference in the PPR before as compared to after LTP in zinc-treated groups. Therefore, copper/LTP-dependent modulation of the PPR in the CA1 region was unique to copper and could not be mimicked by zinc, suggesting the existence of separate mechanisms for the inhibition of LTP and LTP-dependent modulation of PPRs for the two ions.

Next we examined whether AMPA receptor function might be part of the mechanism by which copper inhibited LTP and enhanced the PPR after LTP in the CA1 region. To

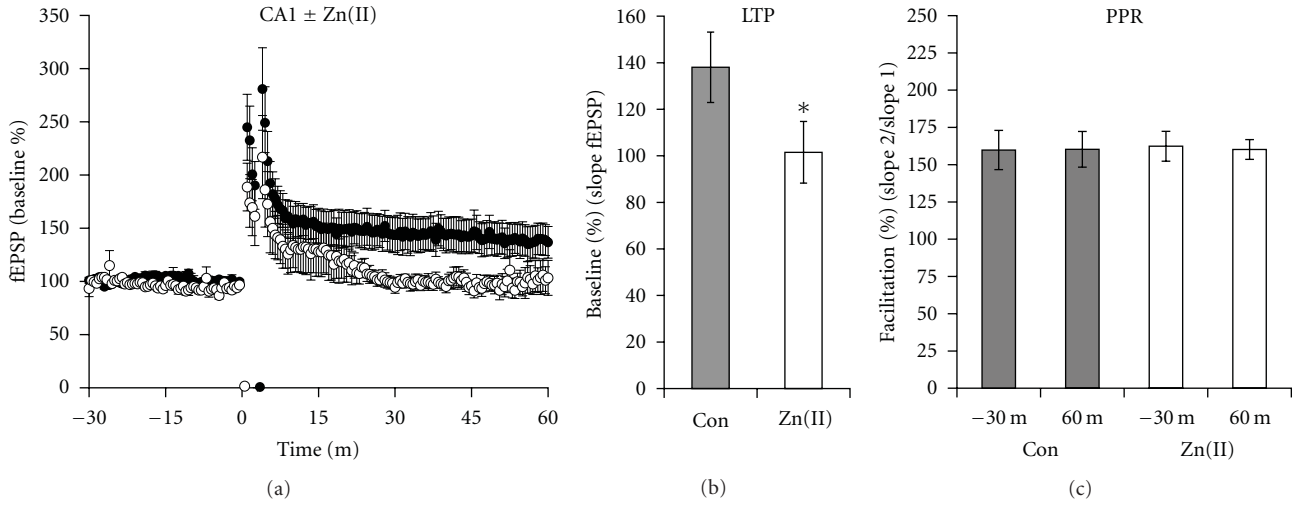

Figure 6: Zinc blocked LTP but did not change the PPR after LTP in the CA1 region. (a) LTP was present in control slices (filled circles) but was blocked by 5 μM $ZnCl_2$ (open circles). (b) The magnitude of the LTPs averaged one hour after the tetanus was reduced from 138.0±15.1% to 101.5 ± 13.2% in zinc-treated slices ($P = 0.013$, $n = 8$). (c) The PPR after the expression of LTP was not affected by zinc (open circles). All slices were tetanized at $t = 0$ m in this experiment.

do this, we treated brain slices with 100 μM CTZ to inhibit AMPA receptor desensitization. We found that CTZ alone did not alter LTP, consistent with previously published results (Figures 7(a) and 7(b)) [29]. However, CTZ enhanced the inhibitory effect of copper on LTP. The enhancement was synergistic since the effect of both copper and CTZ together reduced the LTP by 71.4% while their individual effects added together only reduced it by 43.0% (Figure 7(b)). Presynaptically, CTZ completely blocked, and even slightly reversed, copper/LTP-dependent enhancement of the PPR without significantly altering the baseline PPRs, suggesting that AMPA receptors might be responsible for the presynaptic effects of copper (Figure 7(c)). However, consistent with previous reports, CTZ alone also significantly reduced the PPR after a 90-minute incubation; therefore, its block of the presynaptic effects was not necessarily LTP dependent and instead may have represented an occlusion of the presynaptic effect (Figure 7(c)) [30, 31].

4. Discussion

4.1. Copper Inhibits NMDA Receptor-Independent LTP. Inhibition of LTP by copper has been well established in the CA1 region of the hippocampus, but the mechanism remains poorly characterized. It has been suggested that copper inhibits LTP mainly through a postsynaptic interaction with NMDA receptors; however, many other important effectors of synaptic plasticity are known targets of copper which could contribute to the mechanism [15, 18–20]. To clarify this possibility, we showed here for the first time that copper can inhibit LTP independent of NMDA receptors in the CA3 region of the mouse hippocampus. NMDA receptor independence was ensured by the use of 10 μM MK-801, an NMDA receptor antagonist with an IC_{50} of 0.13 μM, and stimulation of the mossy fiber pathway with the recording electrode placed in the stratum lucidum; a placement that has been shown to isolate NMDA receptor-independent LTP in the CA3 region [23, 32].

The inhibition of LTP by copper in the CA3 region of the hippocampus indicated a possible presynaptic mechanism for copper, as LTP in this region of the brain is thought to be expressed through a presynaptic mechanism [22–25]. Since posttetanic potentiation (PTP) is also a presynaptic phenomenon, this was further supported by our observations that copper reduced PTP [33]. Potential non-NMDA receptor presynaptic targets for copper could include the glutamate release machinery since increased release is a major mechanism behind LTP in the CA3 region [25]. While it has not been replicated in intact cells with physiological concentrations of copper, this idea is further supported by studies showing that copper enhances vesicular binding to membrane fractions [34]. Thus, one could speculate that an interaction between copper- and zinc-binding domains on proteins that regulate vesicular release, such as rab3A, could be a specific target of copper [35, 36]. It is also possible that copper may be interacting with presynaptic voltage-gated calcium channels, GABA receptors, Kainate, or AMPA receptors, as each of these has a role in regulating neurotransmitter release [12, 24, 37, 38].

CA3 neurons are strongly inhibited by GABAergic pathways, and it was necessary to use picrotoxin, a $GABA_A$ receptor inhibitor, in order to obtain LTP in the CA3 region in our studies. GABA receptors serve a complex role in LTP, and there is contradiction in the literature regarding it. For example, in the CA3 region, picrotoxin facilitates LTP, whereas gabazine, used to block presynaptic $GABA_A$ receptors, inhibits it [37, 39]. Additionally, copper was shown to inhibit GABA receptors in whole-cell patch clamp studies but acted as an agonist in brain slices [11, 14]. Therefore, the use of picrotoxin in our studies, although necessary, added

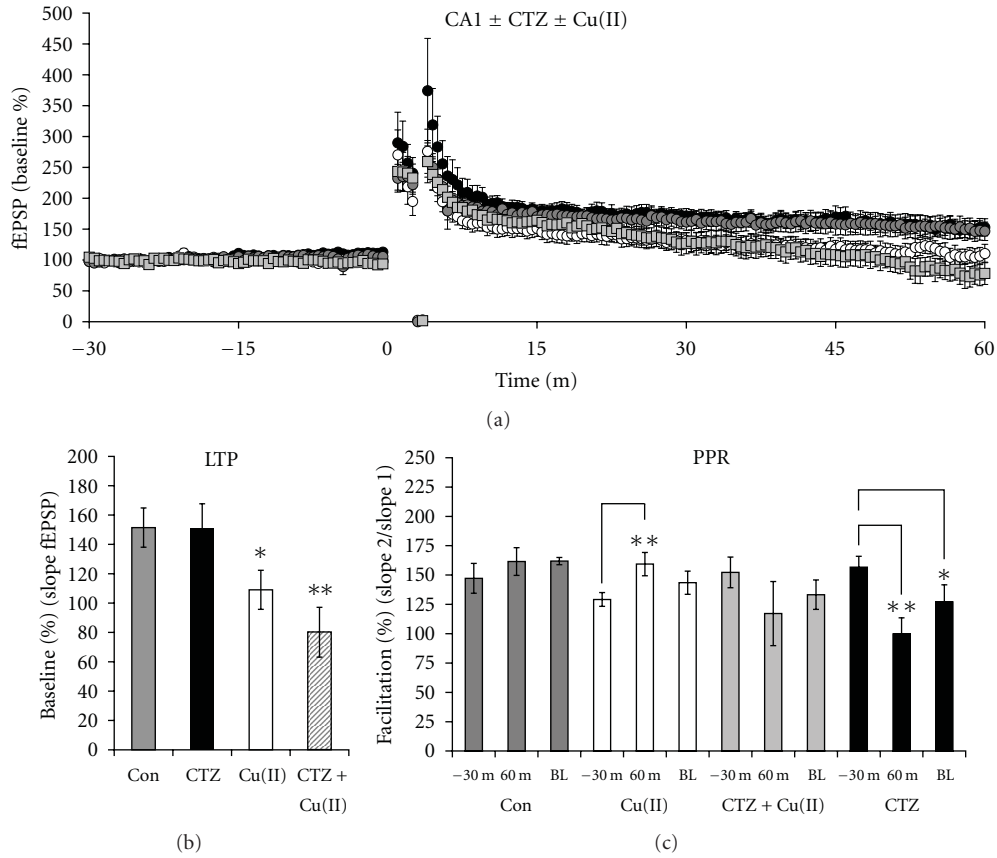

Figure 7: CTZ modulated the effect of copper on synaptic plasticity in the CA1 region. (a) Slices treated with CTZ (black circles) and control slices (gray circles) exhibited identical LTP, but LTP was inhibited by copper (open circles) and by copper combined with CTZ (gray squares). (b) The level of LTP was 151.5 ± 13.4% in control slices (gray bar) ($n = 8$) and 151.0 ± 16.7% in CTZ-treated slices (black bar) ($n = 8$). Copper reduced the LTP to 109.0 ± 13.3% (white bar), and LTP was further reduced to 80.1 ± 17.0% in slices treated with copper and CTZ (striped bar) ($n = 8$). (c) There was no significant difference among the PPRs at 30 minutes prior to the tetanus across groups. At 60 minutes after tetanus, there was no change in the PPR in the control group, a significant enhancement in the PPR in the copper group ($P = 0.028$, $n = 7$), no significant change in the PPR in the CTZ + copper group, and a significant decrease in the PPR in the CTZ group ($P = 0.006$, $n = 7$). The baseline (BL) PPRs measured after 90 minutes without a tetanus was decreased from 152.2 ± 13.1% at 30 minutes prior to the tetanus to 133.3 ± 12.5% in slices treated with CTZ ($P = 0.018$, $n = 7$). The BL PPRs did not vary among the other groups.

complexity to interpreting the potential mechanism of the inhibition of LTP by copper in this brain region. Regardless, the major result of this work was the complete inhibition of NMDA receptor-independent LTP in the CA3 region by copper.

NMDA receptors are known to be inhibited by zinc, and, since copper and zinc share similar biological functions in the nervous system, presumably due to their similar valence, size, and charge, we postulated that copper interacts with this site to inhibit LTP [10, 40–42]. In the CA1 region, we showed that zinc inhibited LTP just as copper did, suggesting that copper and zinc may share this mechanism. However, contrary to our results, it was previously demonstrated that zinc positively modulates LTP [43]. This discrepancy with our work could be explained by differences in the stimulus pattern used to induce LTP, as we used four trains at 100 Hz to induce our LTP, whereas one train of 10–100 Hz was used in the previous study [43]. This explanation was further supported in a follow-up publication showing that LTP in brain slices tetanized six times at 100 Hz was not potentiated by zinc, whereas those tetanized once were potentiated [28]. Thus, increasing the stimulus, strength appears to reduce or, in our case reverse, the enhancing effects that zinc exerts on LTP. This is also consistent with studies showing that the LTP-inducing stimulus pattern effects whether the copper-sensitive Aβ peptide of Alzheimer's disease can inhibit LTP [21]. Finally, it should be noted that we performed our studies at room temperature, whereas those which showed that zinc increases LTP were performed at 26-27°C, and this may have contributed in part to the difference [28, 43]. Overall, our work suggested that copper interacts with the zinc-modulatory site on the NMDA receptor as a potential part of the mechanism for inhibition of LTP by copper in the CA1 region of the hippocampus.

AMPA receptors are a major effector of synaptic plasticity in the hippocampus and are known to be functionally inhibited by micromolar concentrations of copper with kinetics indicative of two binding sites with differing sensitivities to

copper [12, 27]. Therefore, we hypothesized that some aspect of AMPA receptor function might explain the inhibition of LTP by copper in the CA1 region. To investigate this, we treated our slices with cyclothiazide (CTZ), an inhibitor of AMPA receptor desensitization, and measured LTP in our system. Consistent with previous studies, CTZ did not significantly affect or mimic the effect of copper on LTP in these experiments [29]. We interpret this to mean that inhibition of LTP by copper was probably not through a mechanism involving desensitization of AMPA receptors. However, because CTZ was synergistic with copper in enhancing the inhibition of LTP, it was suggested that copper might affect it by a mechanism that includes a component of AMPA receptor function. For example, binding to CTZ might induce a conformational change in the AMPA receptor that increases an interaction with copper.

4.2. Copper Affects Presynaptic Plasticity in an LTP-Dependent Manner. The second major finding of the work presented here is that copper modulated the PPR, a measure of the probability of neurotransmitter release (P_r), in a strictly LTP-dependent manner in both the CA1 and CA3 regions. These results were consistent with a previously published report showing that the PPR is changed in the CA1 region following LTP in rats that had chronically consumed copper in their drinking water [18]. Our results extended these findings to the CA3 region and suggested that LTP induced a change in the presynaptic terminal that creates or unmasks a sensitivity to copper.

The failure of zinc to affect the PPR after LTP in our experiments in the same way as copper ruled out a mechanism such as an interaction between copper and the zinc-binding domain of presynaptic NMDA receptors. Thus, our studies with zinc indicated that inhibition of LTP and LTP-dependent modulation of the PPR were not expressed by the same mechanism and supported the idea that copper influences synaptic plasticity through multiple mechanisms.

Our result showing that CTZ, a compound that blocks AMPA receptor desensitization, completely blocked the effect of copper on the LTP-dependent enhancement of the PPR in the CA1 region suggested that the presynaptic AMPA receptors which regulate neurotransmitter release may be a primary target of copper. However, because CTZ also reduced the PPR in the absence of copper, the apparent block could have resulted from blocking AMPA receptor desensitization to increase the P_r, decrease the PPR, and occlude the copper-dependent increase in the PPR [30, 31]. On the other hand, CTZ and copper could exhibit opposing effects through uniquely different interactions with AMPA receptors. Such opposing effects would be consistent with reports that CTZ enhances AMPA receptor function by increasing AMPA receptor currents and lengthening single-channel opening, while copper inhibits AMPA receptor function by decreasing these currents [12, 44].

An additional aspect regarding the modulation of PPF after LTP was the observation that, in the CA1 region, the PPR was increased by copper, consistent with a mechanism whereby the P_r was decreased. However, after LTP was expressed in the CA3 region, the PPR was decreased, consistent with a mechanism involving an increase in the P_r. If PTX, used in the CA3 region, enhanced the P_r by blocking an antagonistic pathway, this could help explain our result. However, presynaptic $GABA_A$ receptors facilitate neurotransmitter release, and their inhibition blocks LTP in the CA3 region, so presynaptic $GABA_A$ receptors may not have been involved [37]. The P_r is also influenced by the relative sizes of the readily releasable and reserve pools of neurotransmitter, with small releasable pools and large reserve pools supporting paired-pulse facilitation and the converse supporting paired-pulse depression [45]. Thus, the apparent increase in P_r which we observed as a decreased PPR could have resulted from an increase in the readily releasable pool. This would be consistent with a copper-dependent enhancement of vesicular binding to the presynaptic terminal as has been shown in isolated brain synaptic vesicles [34]. Overall, our data clearly shows a copper-dependent modulation of the PPR after LTP in both the CA3 and CA1 hippocampal areas and strongly points to a role for copper in modulating presynaptic plasticity during LTP.

Combined, our work with LTP and short-term presynaptic plasticity suggests potential members of a copper interactome that could include both pre- and postsynaptic NMDA receptors, pre-synaptic AMPA, GABA receptors, Rab GTPases, and voltage-gated calcium channels. In addition, the Alzheimer's disease Aβ peptide, the prion protein (PrP^c), as well as Cu/Zn-superoxide dismutase, are each regulated by binding to copper, have well documented roles in modulating synaptic plasticity, and could be part of a set of copper-interacting proteins that influence the deterioration of learning and memory in neurodegenerative diseases [46–49]. Indeed, copper-based therapies based on an interaction between copper and the Alzheimer's disease Aβ protein are currently in development for the treatment of Alzheimer's disease and show promise for treating prion diseases [49–52]. Thus, the work presented here makes a relevant contribution to our understanding of the mechanism by which copper affects synaptic plasticity and points to its presynaptic involvement in the etiology and treatment of copper-dependent neurodegenerative disorders.

5. Conclusions

We have shown that copper inhibited NMDA receptor-independent LTP in the CA3 region of the mouse hippocampus. Copper had interactions with synaptic plasticity at a presynaptic level, as indicated by our finding that copper significantly enhanced the PPR in the CA1 region and decreased the PPR in the CA3 region in an LTP-dependent manner. In further support of this, copper reduced PTP in the CA1 region and CA3 regions. Thus, LTP caused the appearance of a copper-sensitive factor which modulated the PPR.

Competing Interests

The author has declared that no competing interests exist.

Acknowledgments

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Research Article

Glutamate-Mediated Primary Somatosensory Cortex Excitability Correlated with Circulating Copper and Ceruloplasmin

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Objective. To verify whether markers of metal homeostasis are related to a magnetoencephalographic index representative of glutamate-mediated excitability of the primary somatosensory cortex. The index is identified as the source strength of the earliest component (M20) of the somatosensory magnetic fields (SEFs) evoked by right median nerve stimulation at wrist. *Method*. Thirty healthy right-handed subjects (51 ± 22 years) were enrolled in the study. A source reconstruction algorithm was applied to assess the amount of synchronously activated neurons subtending the M20 and the following SEF component (M30), which is generated by two independent contributions of gabaergic and glutamatergic transmission. Serum copper, ceruloplasmin, iron, transferrin, transferrin saturation, and zinc levels were measured. *Results*. Total copper and ceruloplasmin negatively correlated with the M20 source strength. *Conclusion*. This pilot study suggests that higher level of body copper reserve, as marked by ceruloplasmin variations, parallels lower cortical glutamatergic responsiveness.

1. Introduction

In the last decade, growing evidence has unveiled the involvement of specific metals in brain cortical neurotransmission and the role played by their disarrangements in neurodegeneration. It is well established, for example, that cognitive impairments often follow conditions of metal imbalance, which can be either deficiency, as in Menkes' disease, or accumulation, as in Wilson's disease or aceruloplasminemia [1–6]. Recently, it has been demonstrated that metal dyshomeostases linked to mutations and polymorphisms of specific genes, such as ATP7B in Alzheimer's disease (AD, [7]), the gene of ceruloplasmin in Parkinsons' disease (PD, [8, 9]), the gene of hemochromatosis (HFE) in multiple sclerosis [10, 11] and in AD [12–14], increase the risk of developing those diseases. Copper and zinc have been advocated as primary actors in the neurotransmission of glutamatergic synapses in brain areas that are critical for AD [5, 15, 16]. Iron appears instead mostly involved in dopaminergic neurotransmission [17].

Most of the data on the role of metals in neurotransmission come from studies *in vitro* or animal models, while data in humans are still rather scanty. For this reason, in the current study, we approached the issue in man aiming at assessing whether systemic concentrations of copper, iron, and their related proteins are associated with specific brain indices of cortical glutamatergic neurotransmission.

Glutamate mediates the excitatory neurotransmission in brain networks that are key for sensory perception, memory, and sensorimotor control. In particular, glutamate is the excitatory neurotransmitter of the thalamocortical inputs to the primary visual, auditory, and somatosensory cortices

[18, 19], which makes it crucial for the cortical hierarchic structure controlling the interactions with the environment.

We focused on the primary somatosensory cortex (S1, [20]), since it receives the incoming stimulus via a simple circuit (relayed by only two subcortical nuclei in the brain stem and thalamus) and used a median nerve stimulation protocol. It is known that the earliest cortical response to peripheral nerve stimulation, the M20, results from the glutamate-mediated excitatory postsynaptic potentials (EPSPs, [21–23] generated by area 3b's pyramidal neurons [24–26]. The fact that M20 is highly reliable and is not affected by the subject attention [27] makes its ECD strength a good index of glutamate-mediated excitability in clinical studies, where patient compliance could be limited.

We used MEG to record cerebral activity. Selective sensitivity to electrical currents directed tangentially to the scalp [28] and the cytoarchitectonic distribution of pyramidal cells columns (perpendicular to the cortical surface) make MEG highly sensitive to the activity of area 3b [29–31]. We used localization algorithms to assess the amount of synchronously activated neurons independently of their position. In other words, we wanted the estimate of glutamate-mediated excitability independent of both the recording apparatus positioning with respect to the subject and the individual central sulcus shaping with respect to the scalp.

The aim of our work was twofold: (1) to assess non-invasively in healthy people possible relationships between glutamate-mediated neuronal excitability and metal known to affect this neurotransmission path. (2) To explicate the existence of a noninvasive index of S1 glutamate-mediated excitability (M20 ECD strength), whose reliability will be indirectly strengthened by the ability to assess such relationships.

2. Materials and Methods

The study was conformed with The Code of Ethics expressed in the Declaration of Helsinki and was approved by the Ethical Committee of the "San Giovanni Calibita" Fatebenefratelli Hospital. All subjects signed an informed consent.

2.1. Subjects. Thirty healthy and drug-free subjects (16 females, mean age 51 ± 22, age range [24–93] years) were enrolled in the study. All were right handed with a mean Edinburgh Inventory test score of 83 ± 4 [32]. Neurological history and examination were assessed to exclude sensory deficit. Electroneurographic study of upper extremities was conducted to exclude subclinical deficit of sensitive fibres. Twenty-seven out of the 30 recruited had a complete data set that entered the statistical analysis.

2.2. Biochemical Investigations. Samples of serum from overnight fasting blood were drawn in the morning and rapidly stored at −80°C. Biochemical variables were determined according to established methods reported in details elsewhere [33]. Briefly, serum copper concentrations were measured following the method of Abe et al. [34] (Randox Laboratories, Crumlin, UK) and by an A Aanalyst 300 Perkin Elmer atomic absorption spectrophotometer equipped with a graphite furnace with platform HGA 800. Transferrin [35] and ceruloplasmin [36] were measured by immunoturbidimetric assays (Horiba ABX, Montpellier, France).

Serum iron levels were determined using a Ferene colorimetric method (Horiba ABX, Montpellier, France) [37]. TF saturation (%TF-sat) was calculated by dividing serum iron by the total iron-binding capacity (TBC = TF in mg/dL $*$ 1.25) and multiplying by 100. Zinc level was measured using the specific complexant 5-Br-PAPS [(2-5-bromo-2-pyridylazo)-5-(N-propyl-N-sulfo-propylamino) phenol] according to manufacturer instructions (Zinc, Sentinel Diagnostic, Milan, Italy) (Figure 1).

All biochemical measures were automated on a Cobas Mira Plus analyser (Horiba ABX, Montpellier, France) and performed in duplicate. For each serum copper (total copper) and ceruloplasmin pair, we computed the amount of copper bound to ceruloplasmin (Cu_B) and the amount of copper not bound to ceruloplasmin (free copper) following standard procedures [38]; briefly: Cu_B = ceruloplasmin (mg/dL) $*$ 10 $*$ n; n = 0.0472 (μmol/mg); free copper = total copper − Cu_B). This calculation expresses free copper in μmol/L and is based on the fact that ceruloplasmin contains 0.3% of copper [38]. Thus, for a subject with a serum copper concentration of 17.3 μmol/L and a serum ceruloplasmin concentration of 33 mg/dL, the bound copper concentration = 33 $*$ 10 $*$ 0.0472 = 15.6 μmol/L, and the free copper concentration = 17.3 − 15.6 = 1.7 μmol/L.

2.3. MEG Investigation. Brain magnetic fields were recorded from the left rolandic region by means of a 28-channel MEG system [39] covering a scalp area of about 180 cm^2, operating inside a magnetically shielded room (Vacuumschmelze). Cortical evoked responses were recorded (band-pass filtered 0.48–250 Hz, sampling rate 1000 Hz) during unilateral 0.2 ms long electric pulses (631 ms interstimulus interval) of the right median nerve at wrist, delivered through surface disks with proximal cathode. Stimulus intensity was adjusted until inducing a painless thumb twitch. In this way, all the proprioceptive and the superficial perception fibres are engaged, and neural recruitment is mainly dependent on measurable functional/anatomical circuitry. Moreover, the standardization of stimulus intensity just above motor threshold has been demonstrated to be able to evidence interindividual relationships of primary somatosensory cortical response amplitude with age and gender [40]. The electrical stimulation was delivered to the dominant right median nerve and the cerebral activity recoded contralaterally.

The MEG signals from each of the 28 channels were averaged on the galvanic nerve stimulation at wrist (t = 0, about 300 responses) obtaining the SEFs. The primary cortical responsiveness was estimated by latency, position, and strength of the sources activated in correspondence to the two earliest components (M20 and M30), modelled by equivalent current dipoles (ECDs) within a homogenously conducting sphere, and solution accepted only if explained variance was above 95%.

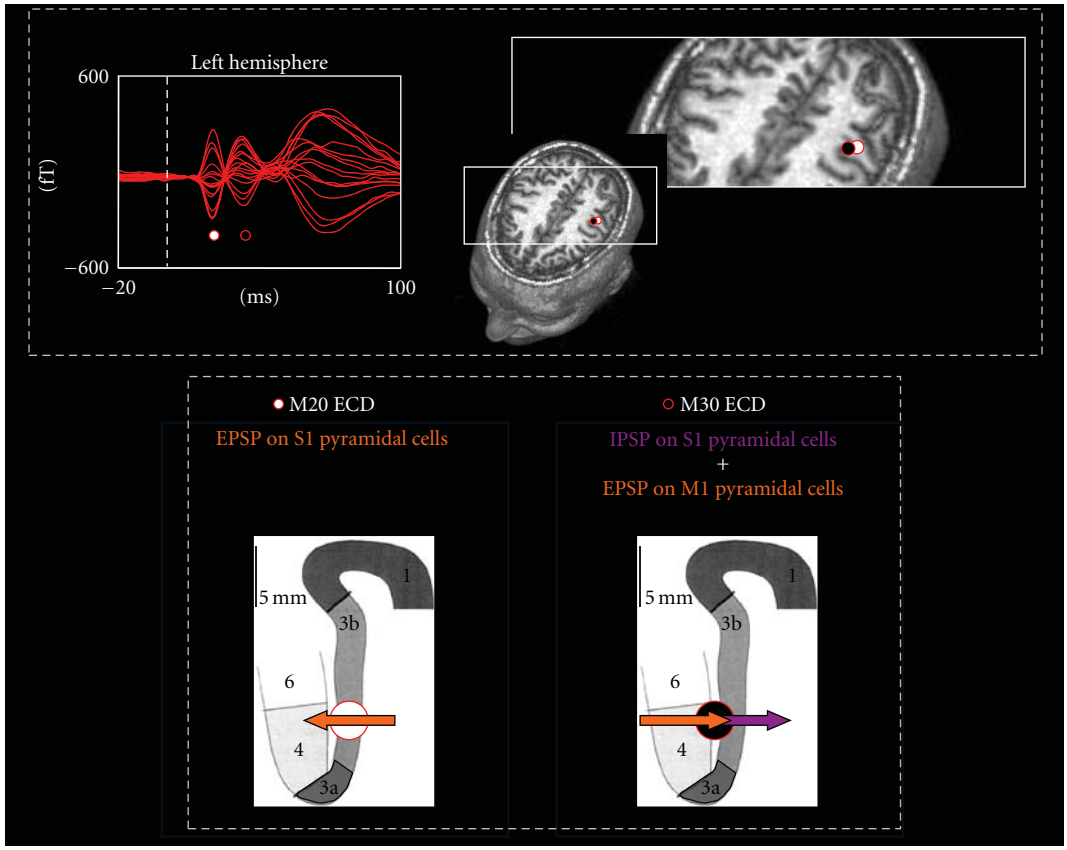

Figure 1: MEG primary sensorimotor cortex excitability indexes. Top left: in a representative subject, the superimposition of all left rolandic region channels in the [−20, 100] ms period, 0 being the moment of the galvanic stimulus arrival to the right median nerve at wrist. Top right: the position of the ECDs explaining the cerebral activation in correspondence to the M20 (white circle) and M30 (black circle) components is projected onto a suitable axial magnetic resonance image slice, through integration on the basis of individual anatomical landmarks. Bottom: schematic representation of currents subtending M20 and M30 generators in a suitable sagittal section of primary sensory and motor areas. Orange arrow indicates current induced by EPSP and purple arrow the effect of IPSP. Circles represent the position of the corresponding equivalent current dipole. The M20 component is mainly generated by EPSPs impinging on pyramidal neurons in area 3b and is mediated by glutamate neurotransmitters. The currents associated with the IPSPs impinging on pyramidal neurons in 3b area are mediated by GABA neurotransmitter and are added to the EPSPs onto BA4 pyramidal neurons to give rise to M30 component.

Our main target was the ECD strength, representing the number of synchronously activated neurons [28], estimated by the localization algorithm in parallel to the ECD position, so that the strength estimate is independent of the source position with respect to the recording apparatus.

2.4. Statistical Analysis. All MEG and biochemical variable did not differ from a Gaussian distributions (Kolmogorov-Smirnov test, $P > 0.200$ consistently).

To make the analysis independent of personal data, correlation with age was checked by computing Pearson coefficients. Gender dependence of M20 and M30 ECD strengths was evaluated by a multivariate analysis of variance with *component* (M20 and M30) as within-subject factors and *gender* (women and man) as between-subject factor. Independent-sample t-test was used to estimate the dependence on gender of the biochemical variables. The association of metal metabolism factors and primary somatosensory neural excitability was studied by correlation matrices between biochemical variables and left M20 and M30 ECD strengths after correction for dependence on age or gender when necessary. Bonferroni correction for multiple comparisons was applied.

3. Results

We controlled MEG indices and biometal variables for gender effects, but none was found (between-subject factor *gender* $P > 0.200$). Thus, descriptive values are provided as a whole (Table 1). M20 ECD strength ($r = 0.403$, $P = 0.027$), iron ($r = -0.478$, $P = 0.009$), and transferrin ($r = -0.431$, $P = 0.014$) were instead correlated for age. Thus, age dependence of M20 ECD strengths, iron, and transferrin levels was considered in all statistical analyses, and partial correlations were computed to correct for the age effect. Positions of either M20 ECD or M30 ECD (Table 2) displayed no dependence on age or gender. No correlations were found between the M20 and M30 strengths ($P > 0.200$).

Table 1: Metals/metal-related proteins.

	Study panel	Reference values
Copper [μmol/L]	13.61 (2.53)	11–24.4
Ceruloplasmin [mg/dL]	26.29 (4.90)	20–60
Free copper [μmol/L]	1.20 (1.96)	<1.6
Zinc [μg/dL]	85.32 (14.75)	68–107
Iron [μg/dL]	98.68 (32.92)	37–164
Transferrin [g/L]	2.76 (0.41)	2–3.6
Transferrin saturation [%]	28.2 (8.4)	12–50%

Mean (standard deviation) of metals and metal-related proteins in serum.

Table 2: M20 and M30 ECD positions and strengths.

	x [mm]	y [mm]	z [mm]	s [nA·m]
M20	−42	−13	69	17.6
ECD	(10)	(11)	(13)	(9.3)
M30	−38	−12	71	21.5
ECD	(9)	(9)	(10)	(16.3)

Mean (standard deviation) position (x, y, *and* z) and strength (s) of left M20 and M30 ECDs. Coordinate system is defined on the basis of anatomical landmarks so that central axis passes through the midline between the two hemispheres, the positive y-axis passes through the nasion and the midpoint between the two preauricular points, and the positive z-axis passes through the vertex perpendicular to y-axes; thus, the positive x-axis is rightward.

The assessment of the biological variables under study indicated that the healthy subjects evaluated in this panel had values coherent with normal reference ranges reported in literature (Table 1). Our statistical analyses revealed that among the biological variables under study, only copper correlated with M20 ECD strength (Table 3). In particular, higher levels of copper corresponded to lower M20 strengths.

We also examined the relation between MEG indices and the two main components of serum copper: ceruloplasmin and free copper (see Section 2). M20 ECD strength did not correlate with free copper, but it displayed a strong inverse correlation with ceruloplasmin (Figure 2), similarly to serum copper (Table 3). Transferrin levels only showed a trend to inverse correlation with the sole M20 ECD strength, even though they did not approach significance (Table 3).

4. Discussion

The pilot investigation presented in this paper aimed at evaluating the involvement of systemic biometal-related variables in human neurotransmission. We focused on a specific brain circuit which connects pyramidal neurons in the somatosensory cortex with a projection coming from neurons in thalamus. Our main result is that in healthy subjects, the strength of M20's ECD, which we deem to be a good marker of glutamate-mediated cortical excitability, is associated with the serum concentrations of ceruloplasmin, which is a marker of copper status.

Table 3: Correlation between SM1 excitability and metals/metal-related proteins.

	M20 ECD strength	M30 ECD strength
Copper	**−0.643** (<.0001)	0.185 (.435)
Ceruloplasmin	**−0.559** (.003)	0.303 (.194)
Free copper	−0.195 (.340)	−0.131 (.560)
Zinc	0.060 (0.808)	−0.015 (0.952)
Iron	−0.134 (0.522)	−0.002 (0.994)
Transferrin	−0.379 (0.068)	−0.065 (0.786)
Transferrin saturation	−0.23 (0.918)	0.080 (0.746)

Correlations (and statistical significance P) between primary sensorimotor cortex excitability and metals or metal-related proteins in serum. Note that correlation values are equal for ceruloplasmin-bound copper and ceruloplasmin (see Section 2). In bold correlation values with $P < .005$ (Bonferroni correction for multiple comparisons).

4.1. Higher Copper Levels Associated with Lower Glutamatergic Excitability. The influence of copper on cortical glutamatergic transmission has been extensively demonstrated at a synaptic level, as copper has been shown to act as a high-affinity NMDA receptor blocker in a voltage-dependent manner [41]. Copper—through the Menkes adenosine triphosphatase (ATPase)—is directly involved in both α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) and N-methyl-D-aspartic acid (NMDA) glutamatergic receptors modulation, resulting in the inhibition of glutamate-mediated neurotransmission [15, 16, 42, 43]. In diverse methodological settings (rat olfactory bulb, [44] long-term potentiation (LTP) in rat hippocampus [45], copper has been demonstrated to be released from copper-containing vesicles in the synaptic cleft postsynaptically, after NMDA receptor activation, causing a depression or a complete blockage of the glutamate-mediated neurotransmission. ATPase7A, also known as the Menkes protein, was shown to be crucial for glutamatergic neurotransmission modulation. This copper chaperone was deemed to pump and accumulate copper into synaptic vesicles, replenishing a pool of copper to be released upon NMDA receptor stimulation. Copper released in the synaptic cleft functionally blocks NMDA receptor limiting Ca^{2+} entry and depolarization of the postsynaptic element [41, 45–47]. Additional copper proteins, such as mettallothiens-3, CuZn superoxide dismutase, and cytochrome, have been reported to modulate glutamatergic synaptic activity and to sustain neurotransmission.

Finally, a similar mechanism was reported in other types of synapses, as, for example, those regulated by the P2X family receptors, which form nonselective channels activated by extracellular ATP. These receptors are widely expressed in the CNS and are involved in synaptic plasticity and in

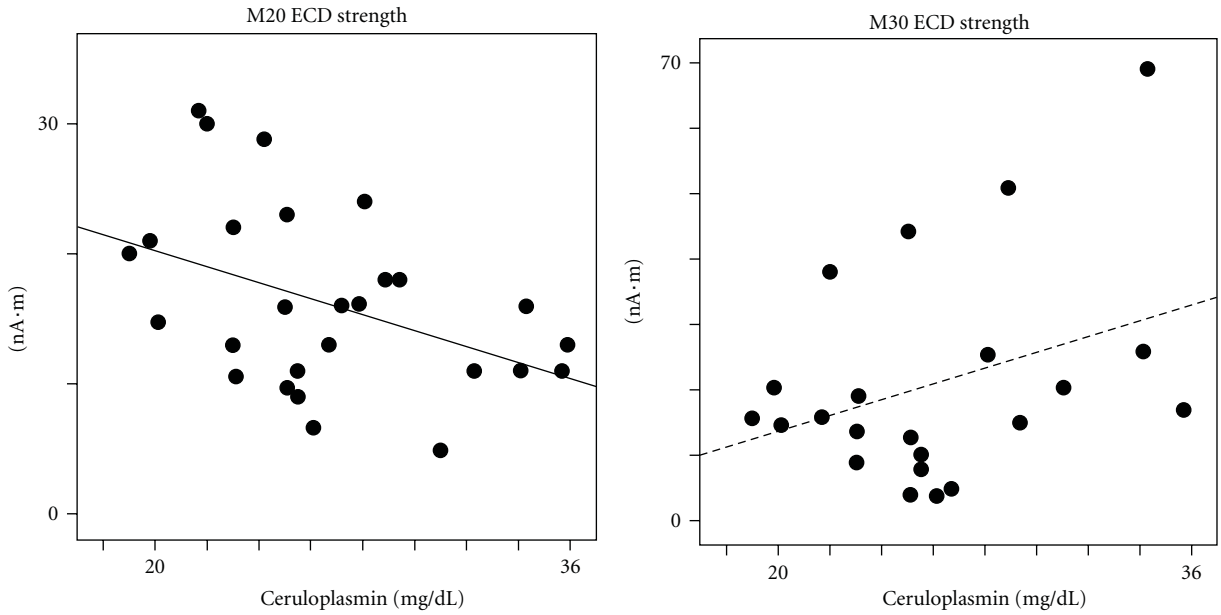

Figure 2: Ceruloplasmin and S1 cortex excitability. Scatter plot of M20 and M30 ECD strengths with respect to individual ceruloplasmin circulating levels. Regression lines are shown for significant (solid line) and nonsignificant (dashed line) correlations.

long-term potentiation. In particular, P2X4 receptor activity is inhibited by Cu^{2+} [48]. On the presynaptic side, prior protein (PrPc, [49]) is involved in regulating the presynaptic Cu^{2+} concentration and synaptic transmission linked to P2X receptors [50].

4.2. Ceruloplasmin and Copper Body Reserve. In normal conditions, about 85–95% of serum copper is structurally bound to ceruloplasmin, whereas the remainder is loosely bound to and is exchanged among albumin, $\alpha 2$ macroglobulin, amino acids, peptides, and several micronutrients and for this reason, is generally referred to as "free" copper [38, 51]. It is generally assumed that ceruloplasmin and free copper are more informative about, respectively, copper bioavailability or copper toxicity than the levels of serum copper. Ceruloplasmin is considered a reliable marker of body copper status since an increase in hepatic copper results in a sustained increase in serum ceruloplasmin concentrations [51–53]. Conversely, copper deficiency results in a ceruloplasmin serum decrease [53]. However, this notion does not hold during infancy, when the liver generates mostly apoceruloplasmin, which is the form of that protein which fails to incorporate copper during its synthesis in the liver, and it is rapidly catabolized [53]. On this ground, ceruloplasmin levels are largely used to monitor the effects of decoppering treatments, as, for example, in zinc therapy, or of chelating agents, as in Wilson's disease, representing a noninvasive surrogate marker of the body copper reserve [6, 54]. Free copper has, instead, been advocated as a superior diagnostic tool to detect copper toxicity, as in Wilson's disease, which is the quintessential example of copper toxicosis or accumulation [38].

4.3. Copper versus Iron: Relationship with Glutamatergic Excitability. If on the one hand copper status variations seem to account for the association of ceruloplasmin-MEG indices, on the other hand iron variations could be also seen as an explanatory reason of this association. In fact, iron metabolism cannot be isolated from copper homeostasis, since there is a crosstalk between copper and iron, represented specifically by ceruloplasmin. Ceruloplasmin is a key enzyme in iron metabolism, which oxidizes Fe^{2+} to Fe^{3+}, thus, facilitating the transfer of cell iron to circulating [55]. In this framework, of particular interest is the cytochrome oxidase [56], which is an iron depending enzyme for energy production in the respiratory electron transport chain of mitochondria, which has been advocated as an endogenous metabolic marker of neuron typology, tightly coupling neuronal activity to oxidative energy metabolism [57]. However, the fact that iron, transferrin (corresponding to the total iron-binding capacity), and transferrin saturation (corresponding to the total bound iron), which are all markers normally used for diagnosis of diseases related to iron metabolism, show no correlation with M20's ECD strength speaks in favor of a primary role of copper in the glutamatergic neurotransmission-ceruloplasmin association.

4.4. M20 ECD Strength as Marker of S1 Glutamate-Mediated Excitability. Certainly, defining M20's ECD strength as a marker of glutamate-mediated neurotransmission implies a simplification. However, while previous evidence sustaining this notion was based on historical cyto- and circuit-architecture [24], more recent data have also been based on pharmacological manipulations of neuronal activities [58]. In particular, the administration of the $GABA_A$ agonist

lorazepam failed to attenuate the M20 strength [58]. Conversely, the strength of M30, which partially relies on GABA neurotransmission as demonstrated by its marked reduction upon lorazepam administration [58], did not associate with markers of copper metabolism in our study. Further investigations by proper pharmacological modulation of glutamate-mediate excitability should be performed to strengthen the concept of M20 ECD strength as noninvasive marker of this neurotransmission system. It is worth noticing that copper density was demonstrated in animal models to be higher in S1 than in all other cortical areas [59], suggesting a possible special role of copper in this primary cortical district. Whether the involvement of copper in glutamate-mediated neurotransmission found in S1 can be generalized to other cerebral regions requires devoted investigations.

To better understand the cause-effect nature of the relationship between glutamate excitability and copper, we plan to act on two levels: (1) in healthy subjects, collecting M20 ECD strength values before and after pharmacological modulation of specific neurotransmission systems (gabaergic transmission by diazepam/lorazepam/zolpidem; less clear how to modulate selectively glutamatrgic transmission, since topiramate, which inhibits glutamate transmission, seems to act also enhancing gabaergic transmission at least at the level of the human motor cortex) and (2) in patients who suffer from an altered copper metabolism, verifying the modulation of the copper-glutamate relationship.

4.5. Glutamatergic Excitability and Copper Status in Man. Human studies of copper homeostasis and of changes in the function of glutamate transporters have been performed in neurological diseases, whereas there is a lack of data from healthy subjects. To the best of our knowledge, a single study used transcranial magnetic stimulation (TMS) to explore cortical excitability and proton magnetic resonance spectroscopy (MRS) to asses metal-related metabolic function in amyotrophic lateral sclerosis [60, 61].

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Review Article

Disturbed Copper Bioavailability in Alzheimer's Disease

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Recent data from in vitro, animal, and human studies have shed new light on the positive roles of copper in many aspects of AD. Copper promotes the non-amyloidogenic processing of APP and thereby lowers the Aβ production in cell culture systems, and it increases lifetime and decreases soluble amyloid production in APP transgenic mice. In a clinical trial with Alzheimer patients, the decline of Aβ levels in CSF, which is a diagnostic marker, is diminished in the verum group (8 mg copper/day), indicating a beneficial effect of the copper treatment. These observations are in line with the benefit of treatment with compounds aimed at normalizing metal levels in the brain, such as PBT2. The data reviewed here demonstrate that there is an apparent disturbance in metal homeostasis in AD. More research is urgently needed to understand how this disturbance can be addressed therapeutically.

1. Copper: Essential and Potentially Toxic

Copper is needed by every oxygen-requiring cell and can be toxic in excess. It is an essential metal with extremely complex roles in numerous different biological functions from acute phase reactant to mitochondrial energy generation. Cu levels are very tightly regulated on the level of duodenal absorption as well as uptake into cells or excretion from cells. Intracellularly, it is transported by specialized chaperone-like proteins to protect the ion from reactions with reactive oxygen species [1].

Plasma Cu levels do not correspond to an individual's Cu exposure or Cu status. Cu serum levels rise during acute phase, they correlate positively with estrogen levels, and can also rise during many chronic disease states. This is caused by an increase in a Cu-containing plasma protein called ceruloplasmin (Cp). Most of the plasma Cu is bound to Cp (up to 95%), with the remaining portion of the Cu being bound to albumin or histidine residues in other proteins [2].

A possible role of Cu in AD has remained a contentious topic during the past 15 years, as has been the question whether extracellular amyloid deposited in plaques is the causative agent in AD. The scientific community was divided as to whether Cu has a role at all, and—if yes—whether it is friend or foe.

Intensive basic research has led to a paradigm shift in both unveiling a beneficial role for Cu in AD on the one hand and deleterious functions of lower intracellular amyloid oligomers consisting of the 42 amino acid residue amyloid-β peptide (Aβ42).

2. Copper and Alzheimer's Disease: Molecular and In Vitro Findings

On a molecular level, interactions between Cu and proteins involved in AD are observed, that is, the amyloid precursor protein (APP), the beta-site APP-cleaving enzyme 1 (BACE1), and the Aβ peptide. APP has a role in Cu-efflux [3] and binds Cu(II) via its extracellular domain [4, 5], where bound Cu(II) is reduced to Cu(I) [6, 7]. Additionally, APP trafficking is directly influenced by Cu. Cu treatment of neuronal cells revealed an increase of APP at the cell surface by both promoting its exocytosis from the Golgi and by reducing its rate of endocytosis [8, 9]. BACE1 is an aspartic protease, cleaving APP in the first step of Aβ generation.

BACE1 binds a single Cu(I) atom with high affinity through cysteine residues of its C-terminal domain and interacts with the cytoplasmic Cu-chaperone CCS (the Cu chaperone for Cu, Zn-superoxide dismutase (SOD1)) through domain I [10]. Aβ, which is part of the ectodomain and the transmembrane segment of APP, was also shown to bind Cu, and Cu was found enriched in plaque deposits which mainly consist of Aβ [11–14]. Thus, APP, BACE1, and Aβ are metalloproteins which can bind Cu and were experimentally shown to be involved in brain Cu homeostasis.

In 1994, the aggregation of synthetic Aβ peptides into assemblies of oligomers and fibrils upon binding of Cu(II) was reported [15]. It was the time before the toxicity of lower Aβ oligomers was discovered and insoluble plaque amyloid was found inert [16]. Over the years, the hypothesis of oligomers as causative agents was confirmed [17]. Recent research has provided evidence that oligomers were shown to induce hyperphosphorylation of tau at AD-relevant epitopes in hippocampal neurons, and thereby provided a strong link between the pathological hallmarks of Aβ and tau deposits [18]. Oligomers isolated from the AD brain were found to potently induce AD-type tau phosphorylation. Tau was found to form Cu-complexes with one Cu(II) ion bound per monomer with a dissociation constant in the micromolar range Cu(II), having an inhibiting effect on the in vitro aggregation of tau [19, 20]. However, since tau occurs intracellularly and Cu(II) is mainly found in the extracellular space, the likelihood that this reflects a physiological function is low.

Findings such that human Aβ directly produces hydrogen peroxide (H_2O_2) by a mechanism that involves the reduction of metal ions, Fe(III) or Cu(II), setting up conditions for Fenton-type chemistry [21] or that Cu(II) and Zn(II) inhibit Aβ fibrillization [22, 23] were all generated by in vitro assays and were never systematically addressed by in vivo model systems.

3. Copper and Alzheimer's Disease: Findings in Animal Models

Maynard et al. have shown that overexpression of the carboxy-terminal fragment of APP which contains the Aβ sequence elicits significantly reduced Cu levels in transgenic mouse brains [24]. Importantly, animal model systems of AD and studies with living cells revealed that APP is actively involved in balancing Cu concentrations. In APP and APLP2 knockout mice, Cu levels were found increased in cerebral cortex and liver [25], whereas overexpression of APP was reported to result in significantly reduced Cu levels in the brain of three transgenic mouse lines [24, 26].

APP23 mice (expressing human APP751 with the Swedish mutation) have amyloidogenic deposits after 6 months and more than half of the population dies before the age of 18 months. Treatment with clioquinol, a hydrophobic low-affinity Cu, and zinc chelator had a significant benefit in this AD mouse model by normalizing, which is elevating, brain Cu and zinc levels. In the treated animals brain, Aβ deposition was decreased, and the general health of the animals was improved [27].

A later study in two different strains of amyloid-bearing transgenic mice confirmed the cognitive benefits of clioquinol treatment [28]. Treatment with clioquinol's second-generation analogue, PBT2, rapidly restored cognition disturbances in AD transgenic mice and was associated with decreased interstitial Aβ [29]. Further, the neurotrophic effects of PBT2 have recently been demonstrated to depend upon metal uptake [30].

Consistent with the above findings, a dietary treatment of APP23 transgenic mice with Cu sulfate for a 3-month interval extended the lifetime of the mice considerably and restored SOD-1 activity back to normal [26]. In agreement with the benefit of Cu treatment, Cu supplementation rescued premature death observed with clioquinol treatment of APP transgenic mice [31]. This is in contrast, however, to the above findings of benefits of clioquinol and PBT2 treatment.

Taken together, these observations indicate that restoring brain Cu homeostasis can have a beneficial effect on disease progression in mouse models for AD. Cu levels can be normalized by dietary treatment with bioavailable Cu salts, or by treatment with clioquinol, which normalizes Cu and zinc brain levels through the formation of metal-ion complexes which are transported across cellular membranes.

In contrast, another animal model, hypercholesterolemic rabbits, showed amyloid-inducing effects mediated by low Cu concentrations in drinking water (0.12 mg Cu/L) [32–34]. This effect was observed when Cu sulfate was added to distilled water, and not when tap water was used in combination with the high cholesterol feed [32]. Potable tap water typically contains at least this amount of Cu (0.12 mg Cu/L), present as Cu carbonate or Cu carbonate hydroxide. Also, when interpreting the findings in the hypercholesterolemic animals, it needs to be kept in mind that high cholesterol levels itself show Aβ-elevating effects [35].

4. Copper and Alzheimer's Disease: Human Trials

Based on the animal study [26], oral intake of Cu(II) in AD patients was investigated in a clinical trial. The efficacy of oral Cu supplementation in the treatment of AD patients was evaluated for 12 months in a prospective, randomized, double-blind, placebo-controlled phase 2 clinical trial in patients with mild AD. Sixty-eight subjects were randomized. Patients with mild AD received either Cu-(II)-orotate-dihydrate (verum group; 8 mg Cu daily) or placebo (placebo group). CSF was collected at beginning and at the end of the study after 12 months. The treatment was well tolerated. The primary outcome measures in CSF were Aβ42, Tau, and Phospho-Tau. Cu intake had no effect on the progression of Tau and Phospho-Tau levels in CSF [36].

While Aβ42 levels declined by 30% in the placebo group ($P = 0.001$), they decreased only by 10% ($P = 0.04$) in the verum group [36]. Since decreased CSF Aβ42 is a diagnostic marker for AD, this observation indicated that Cu treatment had a positive effect on a relevant AD biomarker. There were, however, no significant differences in primary outcome measures (AD Assessment Scale, Cognitive subscale (ADAScog), Mini Mental Status Examination) between the

verum and the placebo group [37]. Finally, CSF Aβ42 levels declined significantly in both groups within 12 months supporting the notion that CSF Aβ42 may be valid not only for diagnostic but also for prognostic purposes in AD [36].

Plasma Cu levels declined only in the placebo group during the 12-month period. However, the outcome of the randomization was such that the placebo group had higher Cu levels at the beginning of the study. Cu levels in the verum group were unchanged, which seems to be paradoxical. One may speculate that Cu treatment normalized Cu levels in plasma in the verum group by enhanced uptake and transport and improved tissue homeostasis.

Previously, we have reported that significantly lower levels of Cu in plasma were found in those AD patients, who fulfilled the criteria of CSF diagnosis for AD [38]. In addition, we observed reduced Cu levels in plasma in patients with higher ADAScog scores (making more mistakes in this neuropsychological test) [39]. However, Cu treatment had no beneficial effect on cognitive abilities in AD patients of the present clinical phase II pilot study [37]. There was no correlation between plasma or CSF Cu and cholesterol levels.

The Cu-clinical trial demonstrates that long-term oral intake of 8 mg Cu (Cu-(II)-orotate-dihydrate) can be excluded as a risk factor for AD, and—based on the CSF biomarker analysis—that Cu may potentially play a beneficial role in this disease.

This is consistent with findings from a pilot phase II clinical trial, where the placebo group deteriorated faster than the clioquinol-treated group suggesting a beneficial effect of clioquinol treatment [40]. In line with these observations, treatment of AD patients with PBT2 in a phase IIa, double-blind, randomized, placebo-controlled trial, PBT2 induced a significant improvement in cognitive performance after 12 weeks without changing plasma Cu levels [41].

Although we found beneficial effects of Cu, there are contradicting results showing that elevated free serum Cu levels (non-ceruloplasmin bound Cu) might be a risk factor for AD [42–47]. A high proportion of the ceruloplasmin of these patients was enzymatically inactive [47], indicating that it contained less-than-normal Cu. Ceruloplasmin is the main Cu carrier in plasma and has many roles, such as ferroxidase or acute phase reactant. However, ceruloplasmin is not a prerequisite for Cu delivery to the periphery [48]. The studies by Squitti, Arnal, and Brewer evaluating levels of non-ceruloplasmin Cu in AD patients do not indicate the chemical nature of this Cu. It is, therefore, unclear whether or not this Cu is readily bioavailable or not. In any case, inactive ceruloplasmin and potentially less bioavailable "free" Cu will directly influence (i.e., lower) intracellular Cu levels and impair the functions of proteins using Cu as a cofactor.

"Free" Cu can have negative effects in the brain as seen in disease states like Wilsons disease. However, levels of Cu in CSF were not upregulated in AD as revealed in a metaanalysis [49]. Instead, the study of Kessler et al. revealed reduced plasma Cu and ceruloplasmin levels in patients with a CSF diagnosis of advanced AD which supports previous observations that a mild Cu deficiency might contribute to AD progression [38].

Whether the observed increase in the non-ceruloplasmin Cu portion is a cause or consequence of the AD pathogenesis also remains to be clarified. The conclusion that Cu intake or exposure can be a risk factor for AD cannot be drawn and a direct comparison between the work by Squitti et al and Brewer et al with an intervention study (8 mg Cu orotate per day, or treatment with PBT2) is difficult.

5. Strategies to Rescue Copper Deficiency: Chelators, Ionophores, and Cu Nanocarriers

APP and APLP2 extracellular domains, but not the extracellular domain of APLP1, decreased intracellular Cu levels in yeast cells and thus possess Cu-efflux activities in this test system [3]. The addition of clioquinol-Cu complexes to the yeast culture medium drastically increased the intracellular Cu concentration, but there was no significant effect on zinc levels. This finding confirmed that facilitated metal-ion transport can act therapeutically by changing the distribution of Cu or facilitating Cu uptake rather than by decreasing Cu levels.

The expression of a mutant APP deficient for Cu binding increased intracellular Cu levels several fold [3]. These data not only uncovered a novel biological function for APP and APLP2 in cellular Cu homeostasis but also provided a new conceptual framework for the formerly diverging theories of Cu supplementation and chelation in the treatment of AD [27]. These results also strictly contradict a proposed metal chelation as a potential therapy for AD that was based on the Aβ interaction with transition metals [50].

Findings described above [3] and in the section of findings from animal models [31] encouraged us to address Cu-deficiency in AD by an aimed facilitation of Cu import into the brain of Cu deficient AD patients. Thus, several parameters have to be taken into consideration, and several requirements must be regarded. The transport and cellular metabolism of Cu depends on a series of membrane proteins and smaller soluble proteins that comprise a functionally integrated system for maintaining cellular Cu homeostasis.

Bypassing the cellular Cu uptake system would be a mean to achieve higher local concentrations compared to normal cellular uptake. We were able to show that synthetic substances called nanocarriers fulfill these requirements. They specifically transport Cu to intracellular regions and enrich local Cu concentrations (e.g. in endosomes versus cytosol) [51]. Cu released from the carrier was bioavailable and compensated decreased Cu levels in living cells. We and others showed earlier that high intracellular Cu levels stimulate the non-amyloidogenic pathway of APP processing, thereby, diminishing levels of toxic Aβ peptides [52, 53]. Vice versa, under conditions of low intracellular Cu, APP proteolysis was shifted from non-amyloidogenic to amyloidogenic processing [52, 54].

Taken together, Cu has beneficial roles in the course of AD based on the following observations: (i) it promotes the non-amyloidogenic processing of APP and thereby lowers the Aβ production in cell culture systems, (ii) it increases lifetime and decreases soluble amyloid production in APP transgenic mice, and (iii) in a clinical trial with AD patients,

the decline of Aβ levels in CSF, which is a diagnostic marker, is diminished. More research is urgently required to understand why there is an apparent disturbance in metal homeostasis in AD and how this disturbance can be addressed therapeutically.

Abbreviations

AD: Alzheimer's disease
APP: Amyloid precursor protein
BACE1: Beta-site APP cleaving enzyme 1
Cp: Ceruloplasmin
Cu: Copper
PBT2: Second-generation 8-hydroxy quinoline analog
SOD1: Cu, Zn superoxide dismutase
ADAScog: AD Assessment Scale, Cognitive subscale.

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Review Article

Copper Modulation as a Therapy for Alzheimer's Disease?

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The role of metals in the pathophysiology of Alzheimer's disease (AD) has gained considerable support in recent years, with both in vitro and in vivo data demonstrating that a mis-metabolism of metal ions, such as copper and zinc, may affect various cellular cascades that ultimately leads to the development and/or potentiation of AD. In this paper, we will provide an overview of the preclinical and clinical literature that specifically relates to attempts to affect the AD cascade by the modulation of brain copper levels. We will also detail our own novel animal data, where we treated APP/PS1 (7-8 months old) mice with either high copper (20 ppm in the drinking water), high cholesterol (2% supplement in the food) or a combination of both and then assessed β-amyloid (Aβ) burden (soluble and insoluble Aβ), APP levels and behavioural performance in the Morris water maze. These data support an interaction between copper/cholesterol and both Aβ and APP and further highlight the potential role of metal ion dyshomeostasis in AD.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that results in the accumulation and aggregation of key proteins within the brain that are believed to drive the symptomatic presentation and pathological progression of the disease. The underlying cause of AD has yet to be elucidated however, there is an increasing burden of proof on the role of metal dysregulation in the pathogenesis of the disease. Other papers in this special edition will detail the proposed role of copper in the generation and aggregation of key AD-related proteins. In this paper, we will review the preclinical and clinical trial data related to attempts to affect the AD cascade by the modulation of brain copper levels. In addition, we will also present our own novel data on long-term copper administration to APP/PS1 transgenic mice (one of the more widely used mouse models of AD).

2. Therapeutic Intervention Studies

In this section, we will outline the various animal and human trials that have been conducted that are specifically directed at a modulation of copper. There are a number of additional studies that will not be discussed, as these assess the effect of therapeutics that may alter a diversity of metals such as copper, zinc, and iron. Keys among these are our own studies with the metal ionophore, PBT2, which has shown promising effects in a number of different transgenic mouse models of AD and also in a phase 2a human clinical trial [1–4]. Its mechanism of action is believed to rely on a normalisation of metal ion (primarily copper and zinc) homeostasis within the brain, which subsequently affects a variety of cellular cascades that, among other activities, serve to facilitate the disaggregation and clearance of beta-amyloid (the principle component of the amyloid plaques that characterises the neuropathology of AD) and also maintains synaptic health

and cognition. While these studies are of great value and have provided impressive data to support this avenue of investigation into the treatment of AD, their mechanisms of action are complex and may not necessarily result from an effect on copper alone.

3. Preclinical Studies

Utilising the APP23 [B6-Tg(Thy1APP)23SdZ] mouse line, Bayer and colleagues [5] treated aged animals (12 months old) with deionized water containing either sucrose or a combination of sucrose and copper sulfate (250 ppm). This copper sulfate treatment, which lasted for three months, was sufficient to elevate copper levels in the APP23 mice (~25% increase), thereby remedying the homeostatic copper deficit present in this transgenic mouse line. The net effect on amyloid burden was a shift to decreased levels of both PBS-soluble Aβ1-40 and 1-42 in male transgenic mice only. Likewise, formic-acid-soluble amyloid burden was only lower in the male animals treated with copper sulfate, while histological amyloid load was decreased in both the male and female transgenic mice treated with copper sulfate. These data are consistent with our own findings, as reported here.

Utilising APP/PS1 mice (B6C3-Tg(APP_{swe}, PSEN1dE9) 85Dbo/J on a B6C3 background (stock#004462, The Jackson Laboratory); 7-8 months of age at the start of the trial; animals develop plaques around 4 months of age), we assessed the effect of either copper supplementation in the drinking water (20 ppm in de-ionised water; $n = 7$), cholesterol supplementation in the food (2% cholesterol in regular rodent chow, provided by Specialty Feeds, Western Australia; $n = 6$) or a combination of both ($n = 4$) on the levels of brain amyloid (control animals received regular rodent chow and regular deionised water; $n = 5$). Treatment was for a period of 16 weeks and during the week prior to culling the animals were assessed for spatial learning and memory in the Morris water maze. In this task, animals were subject to six consecutive days of learning trials (4 trials/day/mouse, 90 seconds/trial with a random quadrant entry and a 15 minute inter-trial interval) and one recall task on the seventh day (submerged platform removed from the pool, one 90 second swimming trial/mouse). We have previously published these techniques [3, 6, 7]. The animals were culled the day after the recall task and the tissues collected (trans-cardial PBS-perfusion followed by removal of the brain, which was immediately dissected into two hemispheres, frozen on dry ice and stored at −80°C prior to analysis). Utilising an in-house antibody (WO2), targeted against residues 5–8 of the human Aβ sequence that detects both full-length monomeric Aβ as well as APP species, as well as a commercial Aβ antibody (4G8, residues 17–21) that detects full-length human Aβ, we assessed Aβ burden by western blot. We have previously published these analytical methods [3, 4, 7] and western blot assessments of Aβ have previously been shown to detect the largest "pool" of this peptide in human samples [8]. Our data (Figure 1) demonstrate similar trends for the effect of the individual treatments, with both the elevated copper and the elevated cholesterol diets causing a trend to

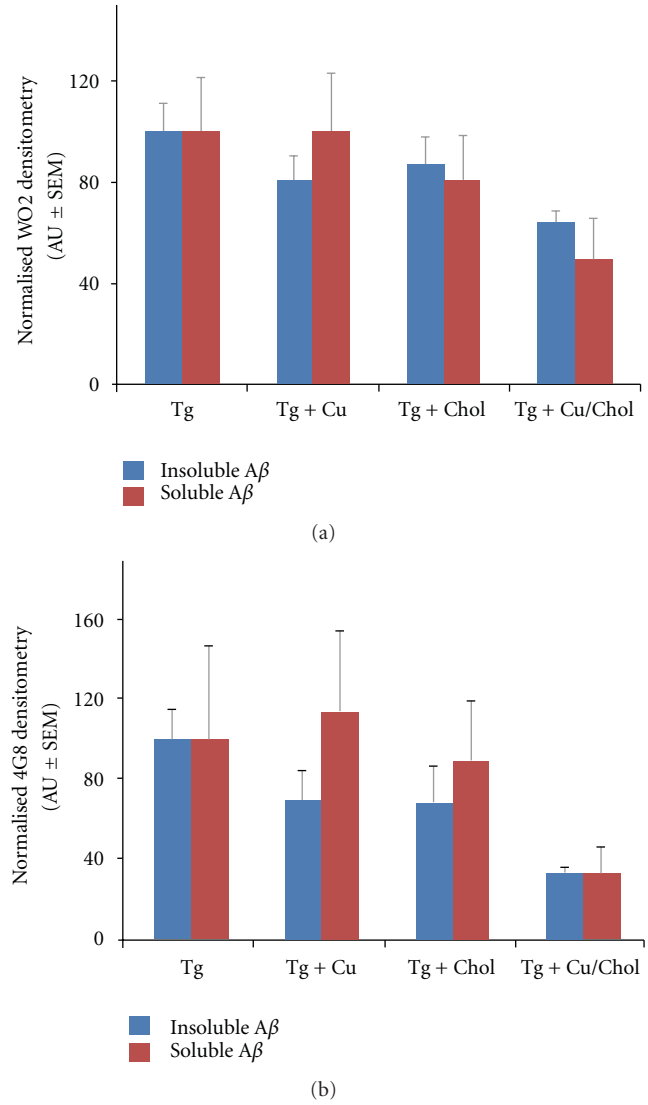

Figure 1: Assessment of Aβ burden in APP/PS1 mice by Western blot. Total hemisphere homogenates (sonicated in PBS followed by centrifugation at 100,000×g for 30 minutes at 4°C and subsequent isolation of the soluble and insoluble fractions. The insoluble fraction was resuspended in PBS prior to a BCA assay and subsequent Western blot) were assessed for Aβ content using both an in-house antibody (WO2) (a) and a commercial (4G8) (b) antibody. A significant reduction in insoluble Aβ was seen with both antibodies for the combined high copper and cholesterol treatment group only, as compared to the Tg control group (Tg).

a decrease in Aβ load. In contrast, a study [9] in a different APP/PS1 model (PS1M146L mice crossed with APPK670N, M671L mice; mice develop plaques at 2-3 months of age) found a trend to an increase in plaque burden in copper-supplemented animals (plaque volume (mm^2) on distilled water alone: 1.63 ± 0.05; on copper-treated distilled water: 1.84 ± 0.05; $P = 0.06$). This study utilised a different start date for treatment (11 weeks of age, corresponding to the start of plaque formation), period of treatment (6 weeks), dose of copper (0.12 ppm copper sulfate) and method of quantitation (histological assessment of Aβ plaque volume

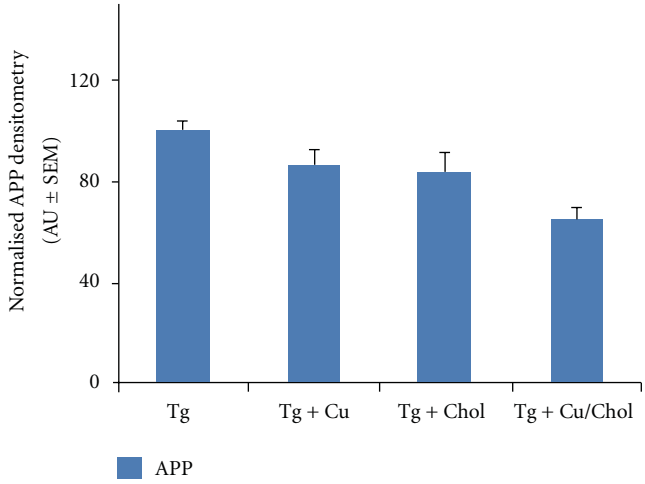

Figure 2: Assessment of APP levels in APP/PS1 mice by Western blot. There is a significant reduction in APP levels in the transgenic mice that received both a high-copper and a high-cholesterol diet, as compared to the Tg control group (Tg).

using the antibody, 10D5) than our own work reported here. These variable experimental parameters are likely to account for the differences in the outcomes observed in both studies.

The copper and cholesterol treatments utilised in our paradigm also appear to be additive, in that there was a significant and exaggerated decrease in Aβ in the Tg animals that received both elevated copper and cholesterol in their diet, as compared to the transgenic animals receiving the control diet (ANOVA: $P = 0.02$ for WO2 pellet/insoluble data, $P = 0.007$ for 4G8 pellet/insoluble data). This effect was consistent across assays with both the in-house (Figure 1(a)) and the commercial (Figure 1(b)) Aβ antibody. These data suggest an interaction of both copper and cholesterol on Aβ metabolism in this transgenic mouse model of AD. Analysis of APP levels (Figure 2) demonstrated that this reduction in Aβ is likely a function of a direct effect on APP, whose protein expression profile closely paralleled that found for Aβ, with a significant reduction in APP in the animals that received the combined high cholesterol and high copper diet (ANOVA: $P = 0.0003$). There was no significant effect of either the copper treatment (ANOVA: $P = 0.09$) or the cholesterol treatment (ANOVA: $P = 0.08$) on APP levels, although a trend to decrease was observed with both. Despite these changes in Aβ levels, there were no significant effects observed in the performance of animals in the Morris water maze task (Figure 3). Repeated measures ANOVA across the six days of learning trials demonstrated a trend to an overall difference between all treatment groups ($P = 0.07$), with a significant treatment x day interaction ($P < 0.05$). Post hoc analysis revealed a significant difference across the trial when comparing the high-copper treatment and the high-copper/cholesterol treatment groups (repeated measures ANOVA: $P = 0.02$) and a trend to a difference between the high-copper and the control transgenic groups (repeated measures ANOVA: $P = 0.07$). Taken together, these data suggest that the decrease in Aβ resulting from a high-copper diet may improve cognitive function, whereas the

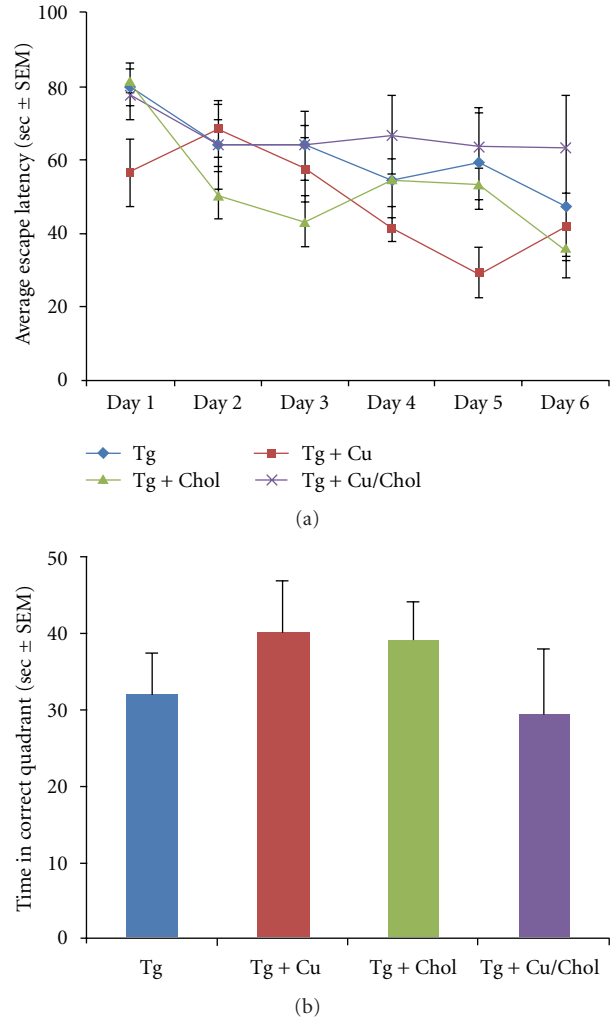

Figure 3: Assessment of spatial learning and memory in APP/PS1 mice in the Morris water maze. There is an overall trend for a modulation of learning performance (a) on this task across the various treatment groups. There was no significant effect on recall in the probe trial across the different treatment groups (b).

combination of both a high-copper and a high-cholesterol diet may negatively impact learning and memory, despite a significant decrease in Aβ burden in those animals. This latter observation, while apparently paradoxical, is consistent with a community-based prospective study that reported that a high-fat diet in conjunction with high-copper intake was associated with a faster rate of cognitive decline in individuals aged 65 years and older [10]. While the mechanisms underlying this have not been pursued, it is likely that an interaction between copper and cholesterol, particularly in the aged brain, may be sufficient to generate unbuffered reactive oxygen species which can contribute to neuronal toxicity and ultimately to cognitive decline [11].

It is also important to note that the effect of elevated copper and/or cholesterol on a "normal" brain may differ markedly to that observed in a "diseased" brain (in which, as is the case in the studies mentioned above, there is an existing homeostatic deficit in copper levels in the brain).

This is apparent from a number of studies where the administration of copper and cholesterol to both rabbits [12] and outbred mice [13] resulted in the accumulation of Aβ and an impairment in various learning/memory tasks.

Thus, more detailed mechanistic investigations into the effect of copper and cholesterol on pathways related both to Aβ metabolism and to cognitive function in both the wildtype and the APP transgenic mouse are required in order to reconcile the differences in these studies.

Following this same principle of elevating copper levels to try and modulate the AD cascade, Phinney and colleagues utilised a genetic approach to achieve this goal [14]. They crossed TgCRND8 mice (a common AD transgenic mouse line that shows a very rapid accumulation of Aβ plaques) with tx^J mice that harbour an autosomal recessive mutation in the gene encoding the copper transport protein, CuATPase7b. The resulting animals (harbouring both mutant human APP and also homozygous for the ATPase7b mutation) had elevated brain copper levels, and the net effect on amyloid burden was similar to that shown in the two studies above, with a decrease in the total brain levels of both soluble- and formic-acid-extractable Aβ (assessed by ELISA) and a significant reduction in the number of histologically identified dense-cored plaques.

The modulation of brain copper levels, therefore, is sufficient to alter the normal generation and metabolism of Aβ in transgenic mouse models of AD. This hypothesis has been further tested in a number of studies that have pharmacologically manipulated copper levels in the brains of APP transgenic mice.

The copper chelator pyrrolidine dithiocarbamate, for example, was given chronically in the drinking water (20 mg/kg, 7 months) to APP/PS1 mice [15] and resulted in a significant increase in brain copper levels, no significant change to amyloid burden, but did improve spatial memory. In contrast, Crouch and colleagues [16] utilised the copper bis(thiosemicarbazone) complex, CuII(gtsm), which elevates cellular copper concentrations and activates various signalling pathways relevant to amyloid metabolism. When administered to APP/PS1 animals for 15 weeks (10 mg/kg/day), there was a significant decrease in PBS-insoluble amyloid, which appeared to be largely driven by a decrease in Aβ trimer species, and a parallel improvement in performance in the Y-maze memory task.

Thus, these preclinical studies suggest that the modulation of brain copper levels may be sufficient to impact the normal pathogenesis of AD. The translation of this work to human studies, however, has not shown the same potential efficacy of this therapeutic approach.

4. Clinical Studies

In the first study of its kind, Squitti and colleagues [17] assessed the effect of the copper-chelating agent, D-penicillamine, ($n = 17$, 600 mg per day for 6 months; $n = 17$ placebo) in a small pilot study in AD patients. A number of cognitive tests were used to assess the effect of the compound, including the Mental Deterioration Battery, MMSE, NeuroPsychiatric Inventory, Geriatric Depression Scale, and the Gottfries Brane Steen scale. While no significant cognitive effects were observed, the placebo-treatment group did not decline as expected, and this precluded any conclusions being drawn on the efficacy of D-penicillamine on cognition (this lack of expected decline in placebo groups has become a recurrent problem in AD trials). Despite this, the data did suggest that there may have been an effect on a number of the biochemical parameters examined. The authors concluded that larger patient numbers were required to further elucidate the potential efficacy of this approach for the treatment of AD.

A more recent clinical trial involved oral copper supplementation in a small cohort of AD patients [18, 19]. Individuals received either placebo ($n = 33$) or oral copper orotate ($n = 35$; equivalent to 8 mg copper per day) for twelve months. The endpoints that were examined included various CSF Aβ species (1-37, 1-38, 1-39, 1-40, and 1-42 species), tau and phospho-tau (thr181) levels and cognitive function, assessed using the Alzheimer's Disease Assessment Scale (ADAS-cog) and the minimental state examination (MMSE). The only biomarker that changed was Aβ1-42 levels, which decreased less (10% drop) in the copper-supplementation group over the course of the study, as compared to the placebo group (30% drop). All other biomarkers and the cognitive scores were unchanged between groups. The implications of the effect seen on CSF amyloid burden are unclear, and the authors concluded that the copper supplementation had no effect on the progression of the AD phenotype.

5. Conclusions

These data highlight the difficulty in translating basic bench science into effective therapeutics. While it is clear that there is a dyshomeostasis in copper levels in the AD brain that may contribute to the pathogenesis of AD (both through a disruption to normal copper-dependent pathways and also via an effect of copper on the aggregation and toxicity of amyloid and formation of plaques) and that in vivo models support the use of compounds that are able to modulate copper levels as being effective at interfering in the normal AD cascades, testing these notions in a human population have thus far been lacking. A definitive understanding of the mechanisms of action and potential interactions of copper in the AD brain are also lacking and are clearly complex. Metals such as copper, for example, are very tightly regulated, and in situations where there is a mismetabolism of copper, a simple dietary supplement is unlikely to change this mismetabolism and may not result in a restoration of cellular copper homeostasis. The "unregulated" delivery of copper to the AD brain may, for example, result in a number of consequences: it may potentiate the aggregation and accumulation of β-amyloid deposits (which arguably may represent either a toxic or a protective phenomenon, depending on ones belief about the role of and interaction between soluble oligomers and insoluble Aβ plaques in the pathogenesis of AD); it may aggravate oxidative pathways to generate toxic ROS or also have other negative cellular effects; it may also activate a number of intracellular copper-dependent pathways that

mediate an improvement in multiple aspects of the pathophysiology of AD, including facilitating the degradation of Aβ and reducing the abnormal phosphorylation of tau (as has been shown when using a more targeted pharmacological approach for the normalisation of copper homeostasis, such as CuII(gtsm) reviewed in [20]). It is clear, therefore, that attempting to modulate homeostatic systems is fraught with difficulties and will require sophisticated approaches to the targeted restoration of metal levels within the brain to ensure that the required outcomes are achieved in the absence of any toxicity. A greater burden of proof, and the identification of a candidate compound, is required to help move this avenue of research forward into robust human clinical trials.

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Research Article

Influence of Water Quality on Cholesterol-Induced Tau Pathology: Preliminary Data

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The studies employed the cholesterol-fed rabbit model of Alzheimer's disease (AD) to investigate the relationship between AD-like neurofibrillary tangle (NFT) neuropathology and tau protein levels as the main component of NFT. We measured brain and plasma tau levels and semiquantified NFT-like neuropathology in cholesterol-fed rabbits administered drinking water of varying quality (distilled, tap, and distilled+copper) compared to animals receiving normal chow and local tap water. Total tau levels in plasma were increased in all cholesterol-fed rabbits compared to animals on normal chow, regardless of quality of water. In contrast, increased tau in brain and increased AT8 immunoreactive NFT-like lesions were greatest in cholesterol-fed rabbits administered distilled water. A substantial decrease in brain tau and incidence and density of AT8 immunoreactive NFT-like lesions occurred in cholesterol-fed rabbits administered copper water, and an even greater decrease was observed in cholesterol-fed animals on local tap water. These studies suggest the possibility that circulating tau could be the source of the tau accumulating in the brain.

1. Introduction

One of the neuropathologic features invariably found in the brains of individuals with Alzheimer's disease (AD) is the amyloid-beta (Aβ) containing senile plaque (SP). Blood samples have been assessed for changes in Aβ levels—driven by the abundant data that Aβ in the brain was the prime candidate for precipitating AD. We showed as did others that circulating levels of the shorter form of Aβ (Aβ40) increased with decreasing cognitive performance within a control population. Levels of Aβ40 were increased even further in mild cognitive impairment (MCI) compared to controls, and circulating levels of Aβ40 are decreased in AD compared to MCI [1]. We suggested that gradually increased production of Aβ occurred with effective clearance from the brain when individuals change from control to MCI and that reduced clearance was evidenced as lower circulating Aβ levels with concurrently increased central accumulation in the transition from MCI to AD [1].

This is consistent with findings in the cholesterol-fed rabbit model of human coronary artery disease where we identified numerous neuropathologic features of AD including central accumulation of Aβ. Early studies were performed in animals administered a diet containing 2% cholesterol and tap water for only 8 weeks compared to animals administered normal rabbit chow and tap water for a similar length of time [2]. Further studies suggested that the induction of AD-like Aβ neuropathology by dietary cholesterol depended on the quality of water the rabbit was drinking. Animals fed 2% cholesterol and drinking distilled water showed minimal AD-like neuropathology, whereas animals drinking tap water were severely affected [3]. Subsequent studies indicated that it was the copper in the tap water that produced the difference in severity of the cholesterol-produced AD-like Aβ pathology [4]. It was clear that cholesterol caused the overproduction of Aβ in the brain and copper influenced its clearance to the blood via inhibition of LRP at the vascular interface [5, 6].

Copper has been implicated in the progression and possibly the cause of AD. Morris et al. have shown that increased copper intake significantly increases the rate of progression of AD in the setting of elevated fat intake [7]. A role for

altered copper metabolism as a cause of neurodegenerative disorders other than AD is clearly recognized [8], and a role for copper in AD is emerging. Ceruloplasmin transports 85–95% of the copper in human blood [8]. Both copper and ceruloplasmin levels have been shown to be elevated in the blood of patients with AD compared to controls by most [9–13], but not all, investigators [14]. Squitti et al. have reported a significant increase in circulating copper in AD and a trend for increased ceruloplasmin [15]. These authors also reported a significant negative correlation in AD between increased copper/ceruloplasmin and decreased scores on the MMSE, AVLT-A7, and the clock draw [15, 16]. Similar to Squitti's data, we have shown that there are significant increases in blood copper/ceruloplasmin in AD compared to age-matched controls [1]. Moreover, circulating copper/ceruloplasmin levels increased in controls with lower performance on the AVLT-A7 and MMSE and remained elevated in MCI and AD as cognitive ability progressively deteriorated [1].

Although found in many other tissues of the body [17], tau protein in brain is the precursor for the other important neuropathologic feature of AD, the neurofibrillary tangle (NFT). Specifically phosphorylated-tau (p-tau) is a main component of the NFT [18]. Single strands of p-tau become paired into filaments called paired helical filaments (PHF), which then assemble into fibrillary complexes within a neuron eventually becoming an NFT. It has been reported that there are increased tau and 181-p-tau levels in MCI subjects converting to AD and that levels are stable in controls and MCI subjects not converting to AD [19]. Although there are reports that the protein tau may have a copper binding site [20–22], there is only one study investigating any relationship between the protein tau and copper in the blood and CSF in AD [16], where a significant positive relationship ($P < .03$) between copper levels in the serum and tau levels in the CSF was identified in AD compared to controls. Nevertheless, there are no reports of changing cognitive performance (i.e., AD versus control) and a relationship between CSF copper and tau levels or copper and tau levels in the circulation.

We recently measured total tau in human plasma and have found significant differences between cognitively normal individuals, subjects with MCI, and patients with AD (unpublished observation). Tau levels (total human tau) were established in each plasma sample using ELISA, kits and standards purchased from Invitrogen Corp., Camarillo, CA. The mean circulating tau level (picograms/mL) is depressed in plasma of patients with AD when compared to both cognitively normal control and individuals with mild cognitive impairment (MCI) ($P < .0001$ and .0002, resp.). The mean tau levels were also significantly reduced in MCI compared to control ($P = .048$), and a significant age-related increase was identified ($P = .042$) in the control population (unpublished observation).

In our previous rabbit studies we administered 2% cholesterol and varying qualities of drinking water, and we were able to manipulate Aβ accumulation and memory but were unable to identify any changes in tau as the major component of the other characteristic lesion in AD-NFT. A recent publication indicated that administering lower levels of dietary cholesterol (1%) over longer periods of time (7 months) could produce NFT-like changes in the brain along with Aβ deposition in female rabbits [23]. An even more recent publication found similar features and behavioral dysfunction in a hypercholesterolemic rat model [24]. Using the same methods employed in the current study, the authors note increased Aβ, tau, and p-tau in the cortex of cholesterol-fed rats [24].

Antibodies have been developed to detect different stages of the NFT formation, including PHF-1 antibody identifying PHF and AT8 antibody highlighting p-tau [18]. Seven months of 1% cholesterol diet caused increased PHF-1 immunoreactivity in hippocampus of female rabbits [23] as well as plaque-like Aβ deposition in the hippocampus and cortical brain regions [25]. We performed a pilot study of female rabbits fed 1% cholesterol and drinking distilled water, copper containing distilled water, or local tap water compared to animals administered normal chow and tap water. In this preliminary study we focus on changes in tau by measuring total tau levels by ELISA and evaluating the brain for the presence of p-tau by performing AT8 immunohistochemistry.

2. Methods

Twenty female New Zealand White (NZW) rabbits approximately 2.5 kilograms body weight were obtained, acclimated to the vivarium for 8–10 days, and assigned to one of four groups. Fifteen of them were fed 1% Cholesterol Diet purchased from Purina TestDiet, the remaining five were fed normal high-fiber chow from Purina LabDiet. The control animals along with five of the cholesterol fed animals were allowed distilled water to drink. Five of the remaining rabbits received local tap water. and the other five were administered distilled water with copper ion added (0.12 PPM copper ion as sulfate). US Filter's analysis of the local Sun City tap water used can be seen in a previous publication [5]. Late in the study, 2 of 5 animals fed the 1% cholesterol chow developed fatty liver disease and/or severe atherosclerosis. These animals were anesthetized and perfused with normal saline as soon as symptoms of either disease appeared. Counter-part animals in the other three groups were sacrificed and similarly processed for tissue collection. Samples collected from these animals have not yet been investigated.

Blood samples were collected prior to initiating administration of the experimental diet and water (baseline). At the end of 21 weeks (5 months) animals were sacrificed after each rabbit was deeply anesthetized with Ketamine (50–75 mg/kg) and Xylazine (5 mg/kg) given intramuscularly in the upper thigh. Additional Ketamine was given at 20% of the original dose, if it was determined that the animal was not deep enough. Once the rabbit was anesthetized, the chest and cranium were shaved, and the animal was placed on an embalming board. An incision was made over the sternum and ribs are reflected to expose the heart. A 23-gauge vacutainer needle was introduced into the left

ventricle, and 10 cc of blood is collected in purple top tubes. The needle was removed and replaced with a 23-gauge 1.5 inch catheter attached to an IV line. The catheter is advanced into the left atrium and secured into place using a vascular clamp. The right atrium is then cut to facilitate drainage using a pair of Mayo scissors. The rabbit was then perfused with a minimum of 120 milliliters of sterile saline using a syringe pump set to deliver 600 cc per hour. Once the animal was perfused, various organs were collected, including the brain, heart, liver, spleen, and kidneys; half of the brain was dissected fresh (frontal pole, whole hippocampus), frozen on dry ice and then stored at −70°C, and the other organs and half-brain were emersion-fixed in 4% paraformaldehyde.

2.1. Tissue Preparation for ELISA Assays. Brain samples were homogenized thoroughly in a cold solution (1 : 8; w : v) of 5 M guanidine HCl and 50 mM Tris HCl, pH 8.0 using a Dounce homogenizer. This solution was transferred to an Eppendorf tube and mixed at room temperature for 3-4 hours. This sample was diluted 1 : 50 with a Dulbecco's phosphate buffered saline with 5% BSA and 0.03% Tween-20, pH 7.4 supplemented with protease inhibitor cocktail (Sigma P8340 or Calbiochem no. 539131) at 1 mM. This diluted sample was centrifuged at 16,000 g for 20 minutes at 4°C prior to use. A fifty μL aliquot of each sample was assessed in the Tau ELISA assay.

Frozen plasma samples were slowly thawed, and a 20 μL aliquot was diluted 1 : 10 with undiluted "standard dilution buffer" provided with each Tau ELISA kit. A fifty μL aliquot of this solution was added to the appropriate well in the following assay.

2.2. Tau ELISA Assay. Samples were assayed using the ELISA kit from Invitrogen (#KHB0042). All reagents used were supplied in the kit including the "standards dilution buffer". Wash buffer was prepared by diluting the supplied concentrate 1 : 25 with purified water. Standards were prepared by diluting the supplied standard with "standard dilution buffer" as specified on labeling to 2000 pg/mL. Serial dilutions were made into standard dilution buffer to 1000, 500, 250, 125, 62.50, and 31.25 pg/mL. 100 μL of standards were added to wells in duplicate. 50 μL of standard dilution buffer was added to the rest of the wells. 50 μL of sample (fresh frozen brain or plasma collected in a purple top vacutainer) was added to ELISA wells in duplicate. The ELISA plate was covered and incubated for 2 hours at room temperature on a rotary shaker (1200 rpm) and thereafter washed 4 times. A 100 μL aliquot of anti-tau detection antibody was added to each well, and the plate was incubated as above for 1 hour and thereafter washed 4 times. Antirabbit HRP concentrate was diluted 100x into HRP diluent and added to wells at 100 μL/well, and the plate is incubated for 30 minutes. After emptying the plate 100 μL of stabilized chromogen was added to each well, and the uncovered plate was incubated at room temperature in the dark for 20–30 minutes on the rotary shaker. The reaction was completed by addition of 100 μL of provided "stop" solution, and the plate was then read at 450 nm in a Bio-Tex ELx800 plate reader. Reader software calculated standard curve, concentration, SD, and CV% (4 parameter algorithm).

2.3. AT8 and Aβ Immunohistochemistry. Blocks of paraformaldehyde fixed tissue were affixed to a metal block with superglue, and 50 μm vibratome sections were collected and stored in 4% paraformaldehyde until use. On the first day, 50 μm thick vibratome tissue sections were washed twice in 0.01 M Tris Buffered Saline (TBS) pH 7.6 for 10 minutes. All steps were carried out on orbital shaker rotating at about 500 rpm. Sections were then transferred to a solution of 3% H_2O_2 in 0.01 M TBS and incubated at room temperature for 30 min. Sections were then washed twice for 10 minutes in 0.01 M TBS + 0.1% triton X-100 pH7.6. Sections were transferred to 88% formic acid for 2-3 min and washed twice for 10 minutes in 0.01 M TBS + 0.1% Triton X-100 pH 7.6 and then transferred to antigen retrieval solution (GeneTex, #GTX28194) for 12–15 minutes. Sections were washed again and then incubated in 3% horse serum in 0.01 M TBS + 0.1% Triton X-100 pH 7.6 for 1 hour. Sections were then washed twice for 10 minutes in 0.01 M TBS + 0.1% triton X-100 pH 7.6 and transferred to 1° antibody (1 : 500 dilution of antihuman PHF-Tau AT8 monoclonal antibody-Pierce Biotechnology or 1 : 100 dilution of 10D5 antibody to Aβ—Elan pharmaceuticals) in 0.01 M TBS + 0.1% triton X-100 pH 7.6) overnight at 4°C. On the following day the sections were washed once in 0.01 M TBS + 0.1% Triton X-100 pH 7.6 and transferred to 2° antibody (Vectastain ABC kit PK-6102 mouse IgG, 1 drop/10 mL 0.01 M TBS + 0.1% Triton X-100 + 1% horse serum) for 1 hour. Then the sections were washed once in 0.01 M TBS + 0.1% Triton X-100 pH 7.6 and transferred to A/B solution (Vectastain kit, 4 drops A +4 drops B/10 mL 0.01 M TBS + 0.1% Triton X-100), washed twice for 10 minutes in 0.05 M Tris pH 7.9 and then developed in a diaminobenzidine solution in dark (15 mg DAB + 20 μL 30% H_2O_2 in 100 mL 0.05 M Tris) for 6–10 minutes. The reaction was stopped by transferring the sections back into 0.05 M Tris pH 7.9. Sections were then mounted, serially dehydrated, and coverslipped.

2.4. Statistics. Mean tau levels ± SD are present. The groups were compared for differences by ANOVA followed by independent t-test. Although mean differences were substantial, the limited number of animals assessed in this preliminary study precluded significance greater than trends ($.05 < P < .10$).

3. Results

We performed a pilot study of long-term dietary cholesterol in four groups of rabbits. One group was administered normal chow and tap water, and three groups were administered 1% cholesterol for five months. The cholesterol-fed groups were administered either distilled water, distilled water supplemented with 0.12 PPM copper ion, or local tap water (routinely containing 0.20 PPM copper ion as well as other trace elements and compounds). Employing methods

Table 1: Tau levels in plasma, whole hippocampus, and frontal cortex, and incidence and density of NFT-like AT8 immunoreactive neurons in the frontal and temporal cortices in rabbits grouped according to diet and water quality. The four groups of rabbits were female rabbits fed (1) normal rabbit chow and provided local tap water to drink (Normal chow-tap), (2) 1% cholesterol diet and distilled drinking water (1% Cholesterol-DW), (3) 1% cholesterol diet and copper supplemented distilled drinking water (1% Cholesterol-DW/Cu), or (4) 1% cholesterol diet and local tap water (1% Cholesterol-tap) for 5 months.

Group	Normal chow-tap	1% Cholesterol-DW	1% Cholesterol-DW/Cu	1% Cholesterol-tap
N	3	3	3	3
Tau level Plasma	24.8 ± 7.7	35.6 ± 3.7	37.2 ± 3.1	34.4 ± 6.8
Tau level Hippocampus	40.4 ± 11.5	216.1 ± 71.3	148.4 ± 48.8	71.8 ± 33.3
Tau level frontal cortex	36.9 ± 15.9	294.2 ± 130.6	62.9 ± 1.9	53.1 ± 32.7
Density of AT8 neurons in frontal cortex	none	abundant	very few isolated	Single faint
Number of animals with AT8 stained Neurons in frontal cortex	0 of 3	3 of 3	2 of 3	1 of 3
Density of AT8 neurons Temporal Cortex	none	Abundant patches of multiple cells	numerous isolated	few isolated
Number of Animals with AT8 neurons in temporal cortex	0 of 3	3 of 3	3 of 3	2 of 3

to measure total tau in human plasma, we measured total tau levels in plasma, hippocampus, and frontal cortex in each animal sacrificed after 5 months of dietary manipulation. We also assessed the hippocampus and frontal and temporal cortex for AT8 staining of p-tau and the frontal and temporal cortex for Aβ accumulation (Figure 1, A4–D4). Plasma samples were taken from each animal at baseline and at sacrifice 21 weeks after initiating experimental diet and controlled diet and water. It is important to note that even though previous studies administered a 1% cholesterol diet and purified water for 7 months [23, 25], we decided to leave open the possibility of identifying enhanced tau pathology in animals on copper containing distilled water and/or tap water and administered experimental diet and drinking water for only 5 months.

We initially had five animals in each of the four groups, but an animal on 1% cholesterol and tap water became ill after 4 months of diet and was sacrificed along with one animal in each of the other groups. After 4.5 months of diet another 1% cholesterol and tap water animal became ill as well as an animal on 1% cholesterol and copper supplemented distilled water—these two animals and a counterpart animal in the other 2 groups were sacrificed. Accordingly there were only 3 animals in each group that completed the 5-month pilot study.

Plasma levels of tau were increased by 40–50% in each of the cholesterol-fed animal groups after 5 months of experimental diet compared to animals on normal rabbit chow and tap water (Table 1). Tau levels in fresh hippocampus were 5-fold higher in animals administered 1% cholesterol and distilled water compared to animals on normal rabbit chow and tap water; levels of tau were 3-fold higher in animals on 1% cholesterol and copper supplemented distilled water and less than 2-fold in animals on 1% cholesterol and tap water (Table 1). A similar graded effect on AT8 staining of p-tau was glaringly apparent in sections of fixed hippocampus (Figure 1). In the fascia dentata of the hippocampus AT8 staining is increased dramatically in animals on cholesterol and distilled water (Figure 1, B1), is less pronounced in the animals on cholesterol and copper (Figure 1, C1), and is further reduced in animals on cholesterol and tap water (Figure 1, D1), all compared to animals on normal chow and tap water (Figure 1, A1). This is equally apparent when viewing the whole hippocampus at low power (Figure 1, 4X; A2–D2). These data support the concept that increasing tau levels are directly related to the severity and intensity of AT8-stained features in the hippocampus. And on the flip side, the data support the premise that our measured levels of tau in plasma and brain tissue are valid and accurate. Further support of this comes from finding similar graded effects when assessing frontal cortex. In the frontal cortex we found a 6-fold increase of tau in animals on 1% cholesterol diet and distilled water compared to animals fed normal rabbit chow. In contrast we found a less than 2-fold increase in tau levels in the frontal cortex of cholesterol-fed animals on copper-supplemented distilled water and tap water (Table 1). Identical to the hippocampus, the number and severity of NFT-like lesions deposits identified in the frontal cortex by AT8 staining correlated to the levels of tau measured (data not shown). AT8 immunoreactive neurons were not observed in the frontal cortex of any animal on normal rabbit chow and tap water. In all of the cholesterol-fed rabbits Becomes administered distilled water to drink, there were abundant AT8 stained NFT-like lesions in frontal cortex (Table 1). The frontal cortex in two of three cholesterol-fed rabbits drinking copper supplemented distilled water exhibited a few AT8 stained cells showing fine processes and considerable arborization (less NFT-like; Table 1). Only one animal fed cholesterol diet and drinking tap water showed a single faintly stained AT8 immunoreactive neuron in the frontal cortex. In the superior temporal cortex no AT8 immunoreactive neurons were observed in animals fed

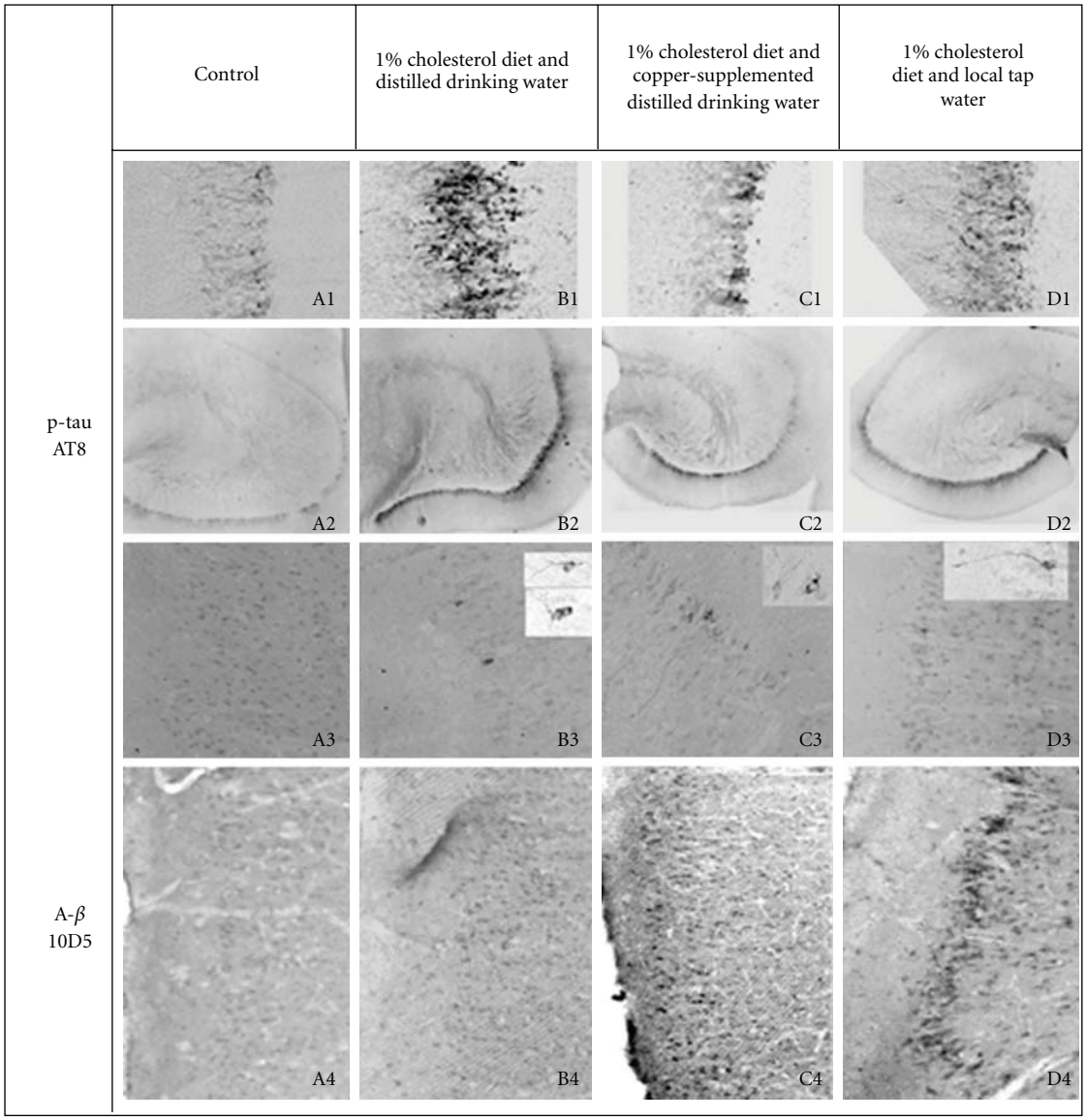

Figure 1: Water quality induced differences in immunohistochemical staining for phosphorylated tau (p-tau, AT8) and amyloid-beta (Aβ; 10D5) in the brains of cholesterol-fed rabbits compared to animals fed normal control diet. The four groups of rabbits were comprised of female rabbits fed normal rabbit chow and provided local tap water to drink (A1–A4), 1% cholesterol diet and distilled drinking water (B1–B4), 1% cholesterol diet and copper supplemented distilled drinking water (C1–C4), or 1% cholesterol diet and local tap water (D1–D4) for 5 months. Representative p-tau AT8 staining in fascia dentate (A1–D1, 10X), hippocampus (A2–D2, 4X), and superior temporal cortex (A3–D3, 10X—insets 40X), and Aβ10D5 staining in superior temporal cortex (A4–D4, 10X) are shown.

normal chow and drinking tap water (Table 1; Figure 1, A3), but 3 of 3 animals fed 1% cholesterol diet and distilled water demonstrated abundant AT8 immunoreactive features which occurred in patches of 2-3 NFT-like lesions (Table 1; Figure 1, B3). All three of the cholesterol-fed animals drinking distilled water supplemented with copper showed numerous isolated AT8 stained NFT-like lesions in the superior temporal cortex (Table 1; Figure 1, C3). Two of three animals fed cholesterol drinking tap water showed very few isolated cells stained with AT8 antibody (Table 1, Figure 1, D3).

Adjacent sections of hippocampus, and frontal and temporal cortex were stained for the presence of Aβ using 10D5 antibody employing the detailed methods. As expected, we found that administration of copper containing distilled water or tap water to drink increased the accumulation of Aβ in hippocampus (not shown) and frontal cortex (not shown) and superior temporal cortex (Figure 1, A4–D4) of animals fed 1% cholesterol diet compared to animals drinking unaltered distilled water, as found previously in rabbit fed 2% cholesterol diet. Similar to findings in animals fed 2% cholesterol diet, we observed a graded effect on Aβ accumulation in animals fed 1% cholesterol diet, with the greatest Aβ accumulation occurring in animals administered tap water (Figure 1, D4), somewhat less accumulation in

animals administered copper supplemented distilled water to drink (Figure 1, C4), while there is minimal Aβ staining in the superior temporal cortex of animals drinking distilled water (Figure 1, B4), all compared to animals administered normal chow and drinking tap water (Figure 1, A4). Overall, there seemed to be an inverse relationship between neuronal accumulation of Aβ and increased levels of tau and associated AT8 immunoreactive NFT-like lesions, based on varying water quality and region of brain. This disconnect suggests that accumulation of Aβ may not cause increased levels of tau, and that once sufficient elevations tau occur in the brain, this in turn leads to deposition of p-tau containing NFT-like lesions.

4. Discussion

This is the first study of the effect of intake of copper or trace metals on levels of the protein tau in the blood and brain. We have shown that there is increased tau neuropathology associated with production of increased tau levels in the blood and brain of the cholesterol fed rabbit. This could be similar to induction of accelerated aging as these findings are consistent with our unpublished observation of age-related increases in plasma tau levels in cognitively normal humans coupled with reported increases in the occurrence and severity of NFT pathology with increasing age in the absence of dementia [26, 27]. The inverse relationship between tau and Aβ pathology depending on water quality is difficult to reconcile when attempting to apply conventional wisdom, where the accumulation of Aβ containing SP is thought to precede the occurrence of p-tau containing NFT in AD and that the processes leading to the formation of the lesions may be linked. What we may have uncovered is that these processes may actually not be linked at all. In this cholesterol-fed rabbit model of Aβ and tau neuropathology, we show that increased accumulation of Aβ occurs concurrently with reduced levels of tau and the incidence of AT8 stained NFT-like lesions. It must be remembered that plaque-only and NFT-only variants of AD have been reported [28, 29]. Neuropathologic evaluation revealed that most clinically diagnosed AD patients had both plaques and tangles, although in the rare circumstance there were individuals who had only senile plaques or only NFT in their brains [28, 29]. Coming full circle there are those who would contend that these individuals were misdiagnosed and those demented patients with only plaques or tangles did not have AD at all (William R. Markesbery, personal communication). This debate continues.

An important feature of animal research is the opportunity to disclose possible mechanisms to explain observations in human studies. Much of disclosing a likely mechanism for altered levels of total tau in plasma in relation to tau concentration in the brain would depend on the origin of the peptide deposited in the brain. If, as all evidence suggests, Aβ accumulates in the brain with increasing change in the vasculature, which in turn leads to reduced clearance of the toxin to the blood [5, 6] and eventually the liver [30], it is difficult to envision how such an alteration in the vascular blood brain barrier (BBB) leads to increased levels of tau and associated formation of NFT-like lesions in the brain—unless the tau accumulating in brain has its origin in the blood. Assuming that 1% cholesterol diet increases tau plasma levels by 40–50% in each cholesterol-fed animal groups, the question is how this similar increase in blood tau has differential effects on tau accumulation in the brain, in terms of tau levels and AT8 immunoreactive NFT-like lesions (5-6 fold increases in the "distilled water" group versus 2-3 fold increases in the "distilled water + copper" group, versus 40% in the "tap water" group)? In other words, the evidence presented would suggest that reduced BBB permeability induced by varying water quality attenuates brain uptake of tau from the blood at the same time it reduces the clearance of Aβ. Therefore, a normally functioning BBB in animals administered cholesterol and distilled water allows clearance of Aβ concomitant with central accumulation of tau due to a concomitant increase in the concentration of tau in the blood. By the addition of copper to the distilled drinking water there is a decrease in BBB permeability leading to either an increased accumulation of Aβ or a decrease in tau levels and associated AT8 immunoreactive features in the brain, while tau concentrations remain elevated to the same level in general circulation. By further reducing water quality by administration of tap water there is even greater compromise in BBB permeability and an even greater accumulation of Aβ and a greater reduction of tau levels and AT8 immunoreactive NFT-like lesions in the brain, again while circulating concentrations of tau remain elevated to the same level. Accordingly it can be proposed that tau deposited in the brain of the cholesterol-fed rabbit model of AD may have come from the circulation. However, it remains unclear how a neuron might take up extracellular tau protein for NFT formation.

Although provocative, one must keep in mind that these are preliminary data and as such require replication. Even so it may be all well and good to be able to induce the production of NFT-like lesions in the brains of cholesterol-fed rabbits, but our eventual goal would be much more important-to find a method or medication to make them go away or not form in the first place.

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Research Article

Association between the c. 2495 A>G ATP7B Polymorphism and Sporadic Alzheimer's Disease

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Nonceruloplasmin-bound copper ("free") is reported to be elevated in Alzheimer's disease (AD). In Wilson's disease (WD) Cu-ATPase 7B protein tightly controls free copper body levels. To explore whether the ATP7B gene harbours susceptibility loci for AD, we screened 180 AD chromosomes for sequence changes in exons 2, 5, 8, 10, 14, and 16, where most of the Mediterranean WD-causing mutations lie. No WD mutation, but sequence changes corresponding to c.1216 T>G Single-Nucleotide Polymorphism (SNP) and c.2495 A>G SNP were found. Thereafter, we genotyped 190 AD patients and 164 controls for these SNPs frequencies estimation. Logistic regression analyses revealed either a trend for the c.1216 SNP ($P = .074$) or a higher frequency for c.2495 SNP of the GG genotype in patients, increasing the probability of AD by 74% ($P = .028$). Presence of the GG genotype in ATP7B c.2495 could account for copper dysfunction in AD which has been shown to raise the probability of the disease.

1. Introduction

There is a general agreement on the existence of a link between Alzheimer's disease (AD) and oxidative stress phenomena triggered by transition metals [1, 2]. The existence of systemic copper dysfunctions in AD has been a controversial issue for many years. In fact, many studies have reported an increase of circulating copper in AD patients with respect to healthy controls [3–13], many others no variation [14–23], and two very recent studies even a decrease of plasma [24] and serum [25] copper in AD patients. Recently, to gain an objective evaluation to the question whether systemic copper variations are associated to AD a meta-analysis of all the studies carried out on serum/plasma copper in AD and healthy cohorts between 1983 and 2010 was run [26]. This analysis demonstrated that AD patients have higher levels of serum Cu than healthy controls. Even though moderate, the assessed copper increase was sufficient to unambiguously distinguish AD patients from healthy controls.

Abnormalities in serum copper not bound to ceruloplasmin ("free" copper [27]) can be advocated as an explanatory variable of copper disturbances in AD [26, 28], as several research groups recently confirmed [3, 25, 29, 30]. Normally, most human serum copper binds tightly to ceruloplasmin [27]. The remaining copper, that is free copper, is distributed and exchanged among albumin, alpha 2 macroglobulin, and low-molecular-weight compounds such as peptides and amino acids (e.g., histidine [31]. A key difference between the two pools lies in the fact that the low-molecular-weight compounds allow free copper to easily cross the Blood-Brain Barrier (BBB) [32, 33]. A recent study confirmed the evidence that the copper transport into the brain is mainly achieved through the BBB as free copper ion, and the blood-cerebrospinal fluid barrier may serve as a main regulatory site of copper in the cerebrospinal fluid (CSF) [34].

Wilson's disease is the paradigmatic example of copper toxicosis or accumulation, in which large amounts of free copper enter the brain and cause cognitive impairment [35, 36], abnormal glial cells and degenerated ganglion cells in cerebral cortex, putamen, and dentate nucleuses [35, 36]. In WD, free copper levels are disproportionately high due to defects in the ATPase 7B (WD protein) and represent most of the circulating copper. Systemic copper abnormalities in AD resemble those observed in WD, though they are very much less severe [10, 37]. Moreover, free copper correlates with the typical deficits [9, 38–40] and markers of AD, namely, CSF Amyloid Beta (Aβ) and Tau proteins [9], as well as an unfavourable prognosis of the disease [40], and tend to predict the annual worsening in Minimental State Examination (MMSE) [10, 41]. WD is an autosomal recessive genetic disorder due to mutations in the ATP7B gene (WD gene), and the rate of occurrence of a single abnormal copy is 1 in 90 people [36]. Based on this, we initiated a hypothesis-driven candidate gene project to determine whether the WD ATP7B gene harbours susceptibility loci for late-onset AD [42, 43]. In particular, in the study presented, we explored the hypothesis that ATP7B sequence changes in exon 2, 5, 8, 10, 14, and 16—where most of the Mediterranean WD-causing mutations lie—have a higher frequency in a group of patients affected by mild or moderate AD compared to a group of healthy individuals.

2. Materials and Methods

190 patients with AD and 164 elderly controls were recruited by two specialized dementia care centres: the Department of Neuroscience, Fatebenefratelli Hospital, Isola Tiberina, in Rome, and the Department of Neurology, Campus Bio-Medico University, Rome, Italy, using a common standardized clinical protocol [10].

The AD patients sample consisted of individuals with a diagnosis of probable AD according to NINCDS-ADRDA criteria [44, 45] and an MMSE score of 25 or less [41]. All AD patients underwent general medical, neurologic, and psychiatric assessments. Neuroimaging diagnostic procedures (magnetic resonance imaging or computed tomography) and complete laboratory analyses were performed to exclude other causes of progressive or reversible dementia. The control sample consisted of healthy volunteers with no clinical evidence of neurological and psychiatric disease. Criteria for exclusion of both patients and controls were conditions known to affect copper metabolism and biological variables of oxidative stress (e.g., diabetes mellitus, inflammatory diseases, recent history of heart or respiratory failure, chronic liver or renal failure, malignant tumors, and a recent history of alcohol abuse).

Among the study populations, 28 AD cases and 41 controls were not analyzed for c.1216 T>G and 10 cases and 13 controls for c.2495 A>G because during the analyses it was not possible to assess the genotype (insufficient DNA/blood sample, sequence analysis failure).

The study was approved by the local IRB, and all participants or legal guardians signed an informed consent.

2.1. SNPs Genotyping. We collected approximately 10 mL of peripheral blood samples from study participants. Genomic DNA from fresh whole blood was prepared using the conventional method for DNA isolation (QLAamp DNA Blood Midi kit).

Polymerase chain reaction (PCR) was performed to amplify the exons and flanking regions of the ATP7B gene. DNA amplification was carried out in a total volume of 25 μL containing 50–100 ng of genomic DNA, 10 pmol of each primer, 0.4 mM of dNTPs, 3 mM $MgCl_2$, and 1 unit of Taq polymerase (Taq Gold, Applied Biosystems) in a thermocycler (2720 Thermal Cycler Applied Biosystem). The conditions were denaturation at 95°C for 30 s, 30 s of appropriate annealing temperature (varying between 53°C–58°C), and 30 s of extension temperature at 72°C for 30 cycles with 5 min at 72°C final extension. Primers, sequences and annealing temperatures are reported in Table 1.

The PCR products that were free of contaminating bands due to nonspecific amplification were column-purified using Nucleo Spin Extract II (Macherey-Nagel). Sequencing PCR reaction was performed in a total volume of 20 μL containing 2 μL Terminator Ready Reaction mix (Applied Biosystems), 3.2 pmol primers, 3 μL Dilution Buffer, 6 ng purified PCR product.

Bidirectional sequencing of exons 2, 5, 8, 10, 14, and 16 of the ATP7B gene was performed using an ABI prism 310 DNA analyzer (Applied Biosystems) with dye-termination chemistry.

Nucleotide changes were detected by comparing the sequence obtained in the chromatogram with the normal gene sequence [NG_008806.1; Homo sapiens ATPase, Cu^{++} transporting, beta polypeptide (ATP7B) on chromosome 13] using SeqScape software version 2.5 (Applied Biosystems).

PCR-restriction fragment length polymorphism (RFLP) assay was applied for detection of c.1216 T>G (rs1801243) ATP7B SNP in AD and healthy controls (Figure 1). PCR-RFLP reaction was the same as the one reported above using specific oligonucleotide primers (Table 1). The T>G transition at the exon 2 creates an MspA1I (Promega) restriction-endonuclease recognition site. The 584 bp PCR product was digested with MspA1I only if the substitution was present. MspA1I reactions were performed at 37°C for 2 h 30 min. All restriction products were analyzed on a 1.5% agarose gel by electrophoresis and visualized by staining the gel using ethidium bromide. Homozygous alleles of the TT genotype appeared as a 584 bp DNA band on the gel, and homozygous alleles of the GG genotype appeared as a 375 bp and an 209 bp DNA band. Heterozygote alleles displayed a combination of the bands (584 bp, 375 bp and 209 bp). Direct DNA bidirectional sequencing was performed for 15% of the PCR products, which were randomly selected and analyzed to confirm the genotypes.

Detection of c.2495 A>G (rs1061472) ATP7B polymorphism was performed by direct bidirectional sequencing of exon 10.

Bidirectional sequencing of exon 10 was performed using an ABI prism 310 DNA analyzer (Applied Biosystem, Foster City CA) with dye-termination chemistry. PCR reaction was

Table 1: Oligonucleotides sequences.

	Primer forward for sequencing	Primer reverse for sequencing	Annealing temperature	PCR product
Exon 2a	5′ AGAGGCCGTCATCACTTATC 3′	5′ CAATGGCAATCAGAGTGGTA 3′	57°C	255 bp
Exon 2b	5′ AGCTCCTAGGGGTTCAAAGT 3′	5′ CAAGGAAAGTTTGCAGGATT 3′	57°C	584 bp
Exon 5	5′ TTTCACAGGCTTTCCTTGAT 3′	5′ ATTTCCATGGGAAAAGTTGA 3′	53°C	336 bp
Exon 8	5′ CGACTGTGCACAAAGCTAGA 3′	5′ CATGGTGTTCAGAGGAAGTG 3′	54°C	386 bp
Exon 10	5′ CAGCTGGCCTAGAACCTGAC 3′	5′ TATCCTCCTGAGGGAACAT 3′	53°C	234 bp
Exon 14	5′ CTGTGCAGGTGTCTTGTTTC 3′	5′ TTTTCCAGACCACACAGAGA 3′	57°C	407 bp
Exon 16	5′ TGTCCTAAAGGATGCTGTCA 3′	5′ GGAAAACAGGCCTGAAATTA 3′	55°C	451 bp
	Primer forward for allele-specific PCR	Primer reverse for allele-specific PCR		
Exon 10	5′ CAGCTGGCCTAGAACCTGACCC 3′	5′ GAAACTTTCCCCCAGGGACCACCT 3′ 5′ ACTTTCCCCCAGGGACCACCC 3′	63°C	141 bp
Beta actin	5′ GTCACATCCAGGGTCCTCAC 3′	5′ CACCTTCACCGTTCCAGTTT 3′	65°C	350 bp

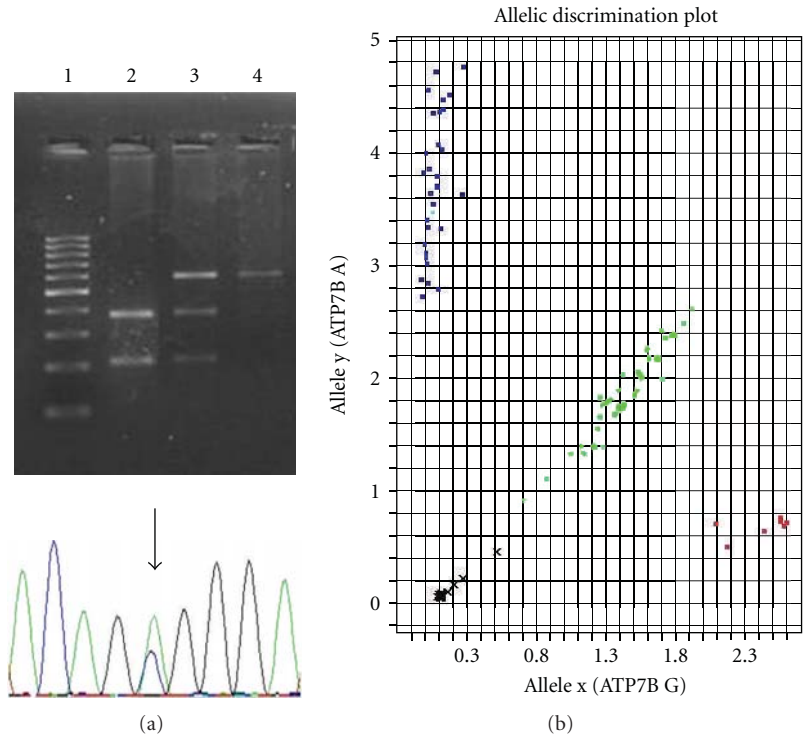

Figure 1: Analytical procedures for ATP7B SNPs detection. (a) PCR-restriction fragment length polymorphism (RFLP) assay for detection of c.1216 T>G SNP. Lane 1 : PCR 100 bp low ladder. Lane 2 : GG genotype (375 bp and 209 bp RFLP). Lane 3 : TG genotype (584 bp, 375 bp and 209 bp RFLP). Lane 4 : TT genotype (584 bp RFLP). In the electropherogram the arrow indicates the TG genotype. (b) TaqMan allelic discrimination assays for detection of c.2495 A>G SNP. Blue: AA genotype. Green: TG genotype. Red: GG genotype; x: undetermined.

the same as the one reported above. Oligonucleotides are detailed in Table 1.

Genotyping of SNPs rs1061472 was achieved by the TaqMan allelic discrimination assays from Applied Biosystems Inc. (Foster City CA) (Figure 1). The predesigned SNPs genotyping assay ID is ID C_1919004_30 (Applied Biosystems). The total reaction volume per well was 20 μL, including 5ng genomic DNA, 1 μL TaqMan SNP genotyping assay (containing two PCR primers and two dye (VIC or FAM)-labelled TaqMan MGB probes) and 10 μL TaqMan Universal PCR Master Mix (Applied Biosystems), according to the manufacturer's manual.

PCR was performed at 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 1 min. The samples were amplified, read, and analyzed using the ABI Prism 7900HT Sequence Detection System and ABI Prism SDS 2.4 software. Two blank controls in each 96-well plate were used for the assay quality control.

Apolipoprotein E (APOE) genotyping was performed according to established methods [46].

Table 2: Demographic characteristics of the investigated groups. Data are mean (SD).

	AD patients	Controls
Number of subjects	190	164
Sex F (%)	70	68
Education mean (SD)	9 (5.0)	9.3 (4.6)
Age (years) mean (SD)	74.4 (7.9)[a]	67.7 (11.2)
MMSE mean (SD)	18.6 (5.8)[a]	28.3 (1.2)
APOE $\varepsilon 4$ frequency(%)	35[a]	13

[*] Correlation is significant at the 0.05 level (2-tailed).
[a]Significantly different between AD and control group ($P < .001$).
[b]Significantly different between AD and control group ($P < .05$).

2.2. Statistical Analyses. Demographic and clinical characteristics in our patient and control samples were described either in terms of mean ± SD if quantitative, or in terms of proportions.

To calculate the power analysis of our study we considered data reported in general population (CEPH) [47, 48] of SNPs allele distribution. As the presence of TG heterozygosis in healthy individuals was about 40% for c.1216 SNP and that of AG heterozygosis was 52% for c.2945 SNP [47, 48], we estimated that, with our sample size, the power was 80% to recognize as significant (at bilateral alpha level of 0.05) a higher prevalence in AD with respect to controls of 12% (or more) for c.1216 SNP and of 11% for c.2945 SNP.

After checking for normality, Student's t-tests were used when appropriate to evaluate differences in quantitative variables. The differences in the overall distribution of the alleles among normal and AD chromosomes were evaluated by χ^2 test. The association of the allele with the largest positive deviation between the observed and the expected frequency under null hypothesis was represented with a 2×2 table and tested by means of χ^2 test. The relative risk of having AD was estimated by Odds Ratios (ORs), and corresponding 95% CIs were also provided. Two-sided χ^2 tests were used to verify Hardy-Weinberg equilibrium. Logistic regression analysis with group (cases and controls) as dependent variable and genetic and demographic measures as independent variables allowed identifying the characteristics more able to discriminate the two groups.

Coefficient pairwise Linkage Disequilibrium (LD; D') between ATP 7B SNPs was estimated using Haploview version 4.2 [49].

All analyses were conducted with SPSS software version 16.0 (SPSS Ltd., Surrey, UK). A P value less than .05 was considered significant in all statistical analysis.

3. Results

Main demographic and clinical characteristics of the subjects participating to this study were reported in Table 2. AD patients and controls did not differ for sex, but differed in age, mean MMSE score, and APOE $\varepsilon 4$ allele frequency (Table 2). As the age effect was considered a potentially confounding factor, it was taken into account in the statistical analyses. As expected, the mean MMSE score was lower in patients than in controls. Education did not differ between the 2 groups, while the presence of at least one APOE $\varepsilon 4$ allele was more frequent in patients than in controls (OR = 3.7; 95% CI = 2.1–6.5; $P < .001$).

The genetic screening for WD mutations in the sole AD cohort by direct sequencing was restricted to exons 2, 5, 8, 14, and 16 of the ATP7B gene in 180 chromosomes, while it was carried out in 360 AD chromosomes and 302 control chromosomes for exon 10. The study revealed no mutations, but sequence changes corresponding to the c.1216 T>G (Ser406Ala) in exon 2 and 2495 A>G (Lys832Arg) in exon 10 SNPs occurred.

The Hardy-Weinberg equilibrium was checked in each group. No statistically significant differences were found.

3.1. C.1216 T>G SNP (Exon 2) in AD and Healthy Controls. Genotype frequencies of c.1216 T>G SNP in our control panel were as follows: TT 30.9%, TG 49.6%, and GG 19.5%. In AD patients they were not different, being TT 24.1%, TG 50.6%, and GG 25.3% ($\chi^2 = 2.25$, $P = .325$). Also allele frequency did not differ between groups (Table 3). When we merged data of TG and TT genotype carriers together and compared their pooled frequency versus that of GG genotype in a model of logistic regression analysis taking into account the age effect, we observed a higher frequency of GG in patients than in controls, although the difference was only marginally significant (OR = 1.773; 95% CI = 0.947–3.320; $P = .074$; Table 3).

3.2. C.2495 A>G SNP (Exon 10) in AD and Healthy Controls. c.2495 A>G genotype frequencies in our control panel were as follows: AA 14.6%, AG 56.3%, and GG 29.1%. In AD patients they were not different, being AA 9.4%, AG 57.1%, and GG 38.9%. The overall χ^2 indicated that the 2 distributions were not clearly different ($\chi^2 = 4.42$, $P = .110$), although the linear component (considering the number of G alleles: 0,1,2) suggested there was an association ($\chi^2 = 4.441$, $P = .036$). G allele frequency was higher in AD than in controls ($\chi^2 = 3.8$, $P = .05$). Furthermore, when we merged data of AG and GG genotype carriers together and compared their pooled frequency versus that of GG genotype, in a model of logistic regression, GG category was significantly more frequent in AD patients than in controls (Table 4). In particular, GG genotype was carried by 39% of AD patients versus 29% of healthy controls and resulted in a significant odds ratio (OR = 1.741; 95% CI = 1.060–2.858; $P = .028$).

Allele frequency of the 2 SNPs in our cohorts (Tables 3 and 4) resembles those reported in HapMap for European origin populations.

To verify whether c.1216 T>G and c.2495 A>G SNPs were in linkage disequilibrium (LD), we constructed plots for our 2 cohorts and compared them with those reported in HapMap database (http://www.hapmap.org/) for European origin population (Figure 1). The analysis revealed that the 2 ATP7B SNPs were not in high LD in our population, either

Table 3: c.1216 T>G ATP7B (exon 2) SNP allele distribution in AD patients and healthy controls and comparison of GG versus TG + TT.

c.1216 T>G ATP7B (exon 2)	AD patients (162)	Controls (123)	*P* value
Allele T frequency n (%)	160 (49%)	137 (55.7%)	$\chi^2 = 2.23$; df = 1; $P = .13$
Allele G frequency n (%)	164 (51%)	109 (44.3%)	
GG n (%)	41 (25.3%)	24 (19.5%)	$P = .074^*$
TG + TT n (%)	121 (74.7%)	99 (80.5%)	

Correlation is significant at the .05 level (2-tailed). *The analyses were corrected for the age effect.

Table 4: c.2495 A>G ATP7B (exon 10) SNP allele distribution in AD patients and healthy controls and comparison between GG versus AG + AA.

c.2495 A>G ATP7B (exon 10) SNP	AD patients (180)	Controls (151)	*P* value
Allele A frequency n (%)	127 (35%)	129 (43%)	$\chi^2 = 3.8$; df = 1; $P = .05$
Allele G frequency n (%)	233 (65%)	173 (57%)	
GG n (%)	70 (39%)	44 (29%)	$P = .028^*$
AG + AA n (%)	110 (61%)	107 (71%)	

Correlation is significant at the .05 level (2-tailed). *The analyses were corrected for the age effect.

when analysing the subjects' sample separately as an AD (D′ = 0.74; D' confidence bounds-Conf. Bounds-0.60–0.84) and a control (D′ value = 0.64; Conf. Bounds 0.5–0.77) cohort or when considering the subjects as a combined population (D′ value = 0.70; Conf. Bounds 0.59–0.79). The degree of LD calculated for the Italian Tuscan population (TSI; D′ = 90; Conf. Bounds 0.77–0.97) and for the Utah residents with Northern and Western European ancestry from CEPH collection (CEU; D′ = 93; 0.85–0.98), on the basis of data reported in HapMap database, was higher than that we identified (Figure 2).

APOE $\varepsilon 4$ and ATP7B (both c.1216 T>G and c.2495 A>G) SNPs were independent AD risk factors, since there was no difference in the frequency of the ATP7B SNPs between carriers and noncarriers of the APOE $\varepsilon 4$ allele (consistently $P > .2$), in addition to when the analysis was restricted to assessment of only the AD population (consistently $P > .2$).

4. Discussion

We have focused the current investigation on ATP7B WD gene, which is a tight control balance regulator for free copper levels in the body [42, 43, 50]. ATPase 7B protein is expressed at high levels in the liver and kidney and at lower levels in the lung, placenta, and brain [50]. It is localized to the trans-Golgi membrane where it maintains intracellular copper concentration by transporting copper from the cytosol across the Golgi lumen. In the Golgi lumen, ATPase 7B mediates the incorporation of copper atoms into ceruloplasmin during its biosynthesis [51–53]. Under elevated copper concentrations ATPase 7B undergoes a reversible, copper-mediated translocation from the trans-Golgi to the apical canalicular membrane where it pumps copper directly into the bile [51–56]. In WD, defects of ATPase 7B prevents

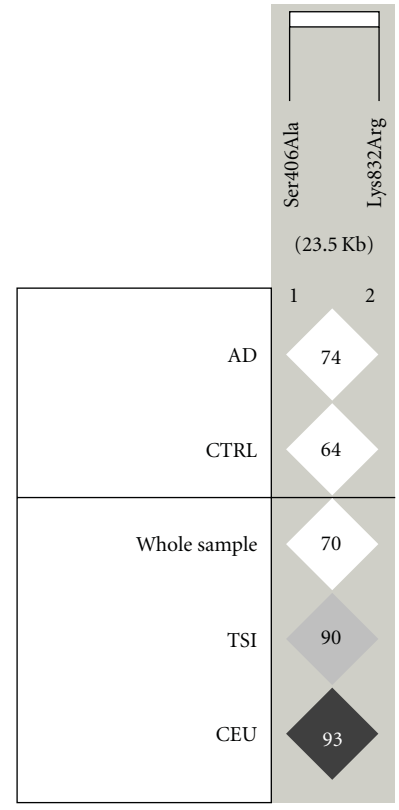

Figure 2: Pairwise LDs are shown, calculated between the ATP7B gene SNPs in AD, controls, whole sample and in HapMap European origin populations (CEU: Utah residents with Northern and Western European ancestry from the CEPH collection; TSI: Tuscans in Italy). The top panel depicts the location of the SNPs in the AT7PB gene. The intensity of the box shading is proportional to the strength of the LD (D′ confidence bounds) for the marker pair, which is also indicated as a percentage within each box.

copper translocation to the secretory pathway as well as the excretion trough the bile, resulting in free copper increased levels and in the secretion of apoceruloplasmin which, being unstable, is rapidly degraded in the blood [54, 57]. In a dedicated study, we have shown that a conspicuous amount of apoceruloplasmin is present in the CSF of AD patients [58]. We have also reported fragmentation of ceruloplasmin, revealed by the presence of low-molecular-weight fragments (<50 KDa) of ceruloplasmin in AD samples from selected patients with higher-than-normal levels of free copper [59].

The most common Mediterranean WD mutations were reported to lie primary in exons 2, 5, 8, 10, 14, and 16 [47, 48, 60–68]. In particular, the Cys271Stop mutation in exon 2 was reported to account for 19% of the total mutations in the European and Turkish population [61]. 1708-1 G>C, 1785 delT, and 1823 del3 have been identified in the Italian population (exon 5) [62–65]. The 2299insC mutation in exon 8 was found in Continental Italians [63]. In the same exon lies the Arg 778 Leu mutation, which is the prevalent mutation of the Mongoloid population [60]. The 2464delC in exon 10 was found in Sardinian and the 2533delA in Sardinian, Continental Italian, Turkish, and Albanian populations [62]. His1069Gln in exon 14, which was reported to account for 17.5% of WD mutations in Mediterraneans, was also found in 20–40% of the WD cases in different Caucasian population groups [47, 63, 65, 66, 68]. Val1146Met and Ile1148Thr in exon 16 have been identified in Greek population [62–65]. Along with these relatively common mutations, several other very rare mutations in Mediterraneans have been described in the exons object of our pilot investigation [62–65].

The ATP7B gene sequence analysis of exons 2, 5, 8, 14, 16 in 180 AD chromosomes—and 662 chromosomes only for exon 10—did not reveal any other sequence change than c.1216 T>G and c.2495 A>G ATP7B SNPs. As a result we focused our study on these 2 SNPs.

Our main observation is that c.2495 A>G ATP7B SNP as either the G allele frequency or the rate of distribution of the GG genotype is higher in AD patients than in healthy controls. The c.1216 T>G SNP was also differently distributed between AD and healthy controls but the significance did not reach the statistical threshold, probably because of the small size of the patient's sample analyzed. However, it has to be noted that the potential role as AD risk factor of the considered ATP7B SNPs could have been masked by the difference of 7 years between our AD patients and controls. In the attempt to reduce this potential confounder we took into account an age effect in our statistical analyses. However, the possibility that some controls might convert to AD while they age another 7 years makes controls and cases more close to each other, and thus our estimate of the statistical association between AD and these SNPs should be considered conservative.

Genotypes and allele distributions for both SNPs found in our panel were coherent with those reported in HapMap for general populations of European origin [47, 48]. While c.1216 T>G and c.2495 A>G SNPs are in LD in TSI and in CEU samples, in our panel they resulted in a lower LD degree.

Exon 2 encodes for a region containing metal-binding domains in ATPase 7B protein. This region encompasses amino acids 1-481. The genetic change in c.1216 T>G SNP corresponds to a substitution ser 406 ala, within this region. Exon 10 encodes for a region within the ATP binding domain region in the protein which encompasses amino acids 820–967. The genetic variation in c.2495 A>G corresponds to a lys 832 arg substitution [43] within this region. Thus it could be argued that these amino acids changes can have an a disturbing effect on ATPase7B function in terms of metal binding properties or ATP hydrolysis which can eventually result in copper homeostasis abnormalities.

This study has a number of limitations which include the small size of the sample, 7 years between cases and controls, the restriction of the sequence analysis to a limited number of ATP7B exons, the need for AD selection with possible sampling bias, and surely it needs confirmation in a larger subject population (in progress). Despite these limitations, this pilot investigation opens new routes—genetic rather than biochemical—for the study of free copper deregulation in AD and strengthens the concept that properly tuning the redistribution of metals via metal complexing or ligand agents, as successfully tested for WD, may positively affect the natural history of AD, at least for the ATP7B c.2495 GG AD carriers [1, 28, 69].

Conflict of Interests

The authors declare no conflict of interests.

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Review Article

Paradigm Shift in Treatment of Alzheimer's Disease: Zinc Therapy Now a Conscientious Choice for Care of Individual Patients

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Breakthrough in treatment of Alzheimer's disease with a shift from irrational dangerous chelation therapy to rational safe evidence based oral zinc therapy. Evidence based medicine: After synthesizing the best available clinical evidence I conclude that oral zinc therapy is a conscientious choice for treatment of free copper toxicosis in individual patients with Alzheimer's disease. Hypothesis 1: Age related free copper toxicosis is a causal factor in pathogenesis of Alzheimer's disease. There are 2 neurodegenerative diseases with abnormalities in copper metabolism: (a) the juvenile form with degeneration in the basal ganglia (Wilson's disease) and (b) the age related form with cortical neurodegeneration (Alzheimer's disease). Initially the hypothesis has been that neurodegeneration was caused by accumulation of copper in the brain but later experiences with treatment of Wilson's disease led to the conviction that free plasma copper is the toxic form of copper: it catalyzes amyloid formation thereby generating oxidative stress, free radicals and degeneration of cortical neurons. Hypothesis 2: Oral zinc therapy is an effective and safe treatment of free copper toxicosis in Alzheimer's disease. Proposed dosage: 50 mg elementary zinc/day. Warning: Chelation therapy is irrational and dangerous in treatment of copper toxicosis in Alzheimer's disease.

1. Introduction

Title of the Study. The title: "Paradigm shift in treatment of Alzheimer's disease: zinc therapy a conscientious choice for care in individual patients" is comparable with the title of the article titled: "Paradigm shift in treatment of Wilson's disease: zinc therapy now treatment of choice" that I published in Brain and Development in 2006 [1]. The title preludes on the idea that lessons learnt from earlier clinical studies on zinc therapy in patients with free copper toxicosis in Wilson's disease have been very helpful in making the conscientious choice for zinc therapy for individual patients with Alzheimer's disease now.

Aim of the Current Review. The purpose of this analytic review is threefold: (1) to cumulate evidence for the hypothesis that Alzheimer's disease caused by an age-related disturbance of copper metabolism (type 2 free copper toxicosis) that leads to oxidative stress and neurodegeneration. (2) to provide evidence for the hypothesis that causal treatment of individual patients with Alzheimer's disease can best be done with antioxidative oral zinc therapy. (3) to warn for the tragic misconception that chelating agents are qualified for treatment of free copper toxicosis.

Alzheimer's Disease and Copper Metabolism. This disorder is a common neurological disease and affects about 250,000 individuals in the Netherlands. Neuropathological research has shown that the plaques detected in the brain of patients with Alzheimer's disease contain deposits of amyloid and abnormal neurofibrils, and the cortical neurons to be withered. The prognosis of Alzheimer's disease is poor. The disease is slowly progressive and spontaneous recovery has never been documented. It is essential to clarify the pathogenesis of the disease, to enable early diagnosis of the disease by means of laboratory testing [2]. As long as the cause of the disease is not exactly known, causal therapy is not possible.

Inflammatory, vascular, and genetic factors are probably important to the disease pathogenesis, but metals, especially "free-metals" like free copper and free iron may be involved

in catalyzing amyloidosis and catalyzing the Fenton's reaction, leading to the generation of free radicals like hydrogen peroxide (H_2O_2) that can damage neurons. Specifically, free copper is an exchangeable pool of copper in serum which is probably in a Cu(I) oxidative state and is loosely bound and exchanged among amino acids, small peptides, albumin, and alpha 2 macroglobulin. This type of copper is intrinsically toxic since it can enter Fenton-like reactions triggering free radical generation [3]. Moreover, for its low-molecular-weight nature it can easily cross the blood-brain barrier as previously [4] and very recently [5] demonstrated in diverse experimental models *in vivo*. In Wilson's disease degeneration upon free copper excess primary hits the liver, the organ which tightly controls metal homeostasis in the body. This is true in cases of severe impairment of the ATPase 7B function, which have usually a juvenile presentation [3]. Free copper toxicosis in AD is sensitively milder, even though very mild effects on AD liver have been reported [6, 7].

A study has shown that the levels of the metal-binding protein metallothionein may be reduced in Alzheimer's disease [8], and another neurodegenerative disease, hepatolenticular degeneration or Wilson's disease is known to be caused by free copper poisoning [9, 10].

While a debate did exist for many years concerning a positive or negative effect of plasma copper on AD, free copper results from clinical studies carried out on Alzheimer's disease patients so far are univocal, demonstrating a detrimental effect of this type of copper on Alzheimer's disease worsening. The works from Squitti and colleagues have shown elevated levels of free copper specifically in Alzheimer's disease, that the free copper disarrangement correlated with worsen clinical status of Alzheimer's disease patients, with a worsen prognosis, and very recently with a status of mild cognitive impairment [11–16]. These studies have been confirmed by other groups from diverse countries [17, 18].

Danzeisen and colleagues [19] reported that free copper is not a suitable marker for copper, apparently questioning results from other authors [6, 13], although no authors ever took into account this possibility. Free copper is, instead, one of the seven diagnostic tests which may help to ensure that the correct diagnosis of Wilson's disease is made [3, 9]. On the other hand, copper concentration in the liver is the most robust marker of copper status in the body, but the measurement is invasive. Ceruloplasmin can be a quite good marker of body copper status, especially in condition of a copper deficiency, as for example on a copper-deficient regimen, which results in ceruloplasmin serum decreases [20].

2. Neurodegeneration and Copper Metabolism

There are many neurodegenerative disorders, such as Alzheimer's disease, lenticular degeneration in Wilson's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, age-related macular degeneration, and hereditary ataxia. The pathogenesis of most of these diseases is unknown. Wilson's disease is the only exception: neurodegeneration of the basal ganglia of the brain is caused by free copper toxicosis [3, 10]. Wilson's disease became the first neurodegenerative disease for which causal treatment was discovered. Causal therapy is possible: zinc therapy is a safe and effective treatment of free copper toxicosis [21].

3. Amyloidosis and Free Copper

The characteristic Alzheimer plaques contain accumulations of amyloid. These deposits also have excess copper and iron. Research into development of drugs for Alzheimer's disease target at amyloid accumulation in the brain. Amyloid is also found in the macula of patients with age-related macular degeneration. Zinc supplementation aiming at decrease of oxidative stress has been found to be effective [22].

Amyloidosis in Type 2 Diabetes Mellitus. amyloid deposits accumulate in plaques in pancreatic cells, a process that is catalyzed by free copper. Hydrogen peroxide is generated during the aggregation of amylin peptide into amyloid. The formation of hydrogen peroxide is greatly stimulated by free copper and could cause progressive degeneration of islet cells in type 2 diabetes mellitus [23].

4. Chelation Therapy in Wilson's Disease

Initially, Wilson's disease was thought to be caused by toxic effects of copper deposits that had been accumulated in the basal ganglia, and metalchelators such as BAL and penicillamine were given in order to liberate copper from the stores and increase the excretion of copper via the urine [9]. Indeed for about 50 years penicillamine has been used for the treatment of copper accumulated in Wilson's disease, and the toxic chelating agent became accepted worldwide as the treatment of choice: survival was thought to be prolonged by penicillamine and signs of copper accumulation, such as Kayser-Fleischer rings, diminished or disappeared [9].

The Chelation Therapy Fallacy. Evidence-based medicine disputes efficacy, safety, and much of the theory behind chelation therapy. The fatal flaw of the copper chelating strategy is that it is based on the method of induction and that it was developed without any deductive, evidence-based reasoning and without any clinical trials having been performed.

5. Chelation Therapy in Alzheimer's Disease

An article on nanochelationtherapy published in September 2005 [24] first drew my attention to the disturbance of copper homeostasis in Alzheimer's disease. The authors referred to two articles published by Squitti and colleagues [10, 11]. In one of these studies, Squitti et al. [25] had measured serum copper concentrations in 47 patients with Alzheimer's disease, 24 patients with vascular dementia, and 44 healthy controls. Mean free copper concentrations in the Alzheimer's disease patients were significantly higher than in the vascular dementia patient group and control group. The authors speculated that the raised copper concentrations could play a role in the pathogenesis of the degenerative process. In an

earlier study, the same authors had described the results of a double-blind, placebo-controlled pilot study of the effects of chelation therapy with penicillamine (600 mg/day) for 6 months in patients with Alzheimer's disease [25]. At that time, it was not appreciated that patients could be suffering from free copper poisoning. Instead, treatment was focused on copper accumulation, and the aim of treatment was to increase the excretion of copper in urine, to reduce laboratory measures of oxidative stress, and to slow the progression of cognitive deterioration, as measured with neuropsychological tests. Thirty-four patients took part in the study: 17 received placebo and 17 received penicillamine. Only 9 patients in both groups completed the study. Of the patients treated with penicillamine, 1 died of a heart infarct and 4 experienced serious adverse events. The pilot study was stopped prematurely by the ethics committee because there were too many adverse events. The authors concluded that penicillamine had not slowed the clinical progression of the disease and that a less harmful chelating agent should be sought. As far as I am aware, Squitti and her colleagues are the first investigators to have performed a controlled clinical study on efficacy and side effects of chelation therapy.

6. Evidence-Based Medicine in Alzheimer's Disease

Making decisions on causal drug therapy in individual patients with Alzheimer's disease is hampered by the fact that randomized clinical trials to base the decisions on are not easy to find. Nevertheless such trials are essential to identify dangerous and worthless treatments. I found that he clinical trial described by Squitti et al. in 2002 [25] is such a trial. It was a real eye opener, it identified the paradoxical effect of penicillamine in Alzheimer's disease: the trial had to be stopped by the ethical board because of the severe side effects of the chelating agent. This paradoxical effect of penicillamine in Alzheimer's disease reminded me of the dangerous paradoxical effect seen in patients with free copper toxicosis in Wilson's disease and it stimulated me to develop the hypothesis that free copper toxicosis might play a causal role in Alzheimer's disease.

In my opinion, chelating agents, including PBT1 (clioquinol) and PBT2 [26, 27], should be tested no more on their effect on safety in treatment of patients with Alzheimer's disease. An ethical committee involved in the preparation of such a clinical trial should not give permission for it being performed. Since we know that copper metabolism is disturbed in Alzheimer's disease and since we know that a randomized clinical trial with the chelating agent penicillamine had to be stopped by the ethical committee because of severe side effects [25], it is irresponsible to ask patients to give permission to participate in a clinical trial with the chelating agent clioquinol.

7. Zinc Therapy

A randomized clinical trial testing a clear hypothesis is needed before conclusions can be drawn about the value of zinc supplements in the treatment of Alzheimer's disease. Such a clinical trial could be set up on lines similar to the randomized clinical trial of penicillamine performed in 2002 [25]. The effect of a low dose of zinc (50 mg/day) on the free copper concentration in serum, the urinary excretion of copper, laboratory markers of oxidative stress, and cognitive functions could be investigated in a blind, placebo-controlled trial. In conclusion, the work of Squitti et al. has provided data to justify the hypothesis that free copper concentrations are raised in patients with Alzheimer's disease. In my opinion, it is justified, and even desirable, to test in a controlled study the hypothesis that the free copper poisoning of patients with Alzheimer's disease is amenable to zinc therapy. A previous study suggested potential benefit of a zinc therapy in AD [28]. Moreover, a very preliminary study with Zinc therapy gave positive results in counteracting AD progression [28]. Specifically, a previous study suggested potential benefit of a zinc therapy in AD [28]. Ten patients were treated, all of them receiving 50 mg of oral zinc *bis*-(dlhydrogen aspartate) three times daily obtaining improvement of memory, understanding, communication, and social contact in eight patients, as stated by the author [28]. The discontinuation of the treatment decreased and even reversed the improvement, in all patients. However, these conclusions have to be taken very cautiously. Even though these limitations, a phase II, multicentre, prospective, randomized, double-blind, placebo-controlled, parallel group study conducted with patients presenting a diagnosis of mild to moderate clinical trial with the following characteristics: (a) a treatment duration of at least one full year, as expected with curative compound use versus symptomatic approaches; (b) a patient-inclusion criterion based on individual serum Cu-dysfunction evidence; (c) monitoring of Cu bioavailability throughout the study with detection of Cu metabolism markers such as free Cu, ceruloplasmin, or Cu/Zn superoxide dismutase levels, to prevent adverse events; (d) statistical power (at least 75 patients per arm, placebo and treated); a dose of zinc sulphate 400–1200 mg/day, can be proposed.

8. Additional Paragraph on Very Recent Studies on Alzheimer Research

Two very recent articles [29, 30] cast biometals in the scenario of the variegated milieu of biomarkers of cerebrospinal fluid (CSF), imaging biomarkers and peripheral biomarkers of Alzheimer's disease under investigation. In recent years, concentrations of CSF amyloid-β42, total Tau, and phosphorylated Tau, *in vivo* molecular imaging of intracerebral beta-amyloid load (by the Pittsburgh Compound-B: PiB-PET), structural and functional neuroimaging changes, or peripheral biomarkers (including inflammatory markers interleukins, cytokines, oxidative stress compounds isoprostanes, plasma APP markers (BACE 1) and other markers of synaptic damage/neurodegeneration) [31–33] have been repetitively reported as differentiating factors of Alzheimer's disease which also reflect core pathological changes of the disease, eventually leading to full dementia. The two papers, which disclose the potentiality of metallochemistry in clinical

studies on Alzheimer's disease patients [29, 30] have been preceded by numerous and heterogeneous reports exploring the reliability of some metals, particularly copper, in characterizing Alzheimer's disease patients and cognitive worsening. Besides the so many case-control studies evaluated in a recent meta-analyses by Bucossi and coworkers [29], at least two prospective studies did demonstrate the relevant influence of copper dysfunction of cognitive status. Specifically, (i) a community-based prospective study exploring cognitive functions in a cohort of 3,718 elderly individuals [34], revealed that a diet high in copper combined with a high dietary intake of saturated fats associated with a faster rate of cognitive decline, specifically with a lost cognition at a rate three-times higher than expected [34]; (ii) a study on 81 subjects with mild to moderate Alzheimer's disease patients, clinically followed up for 1 year[15] finding that higher levels of copper at the baseline correlated with a worsened cognitive status at 1 year. These results are in line with the Rancho and Bernardo study [35] which evaluated 602 men and 849 women for metals (Cu, Fe, and Zn) in association with cognitive performance and revealed that women had worsened performance in total and long-term word (but not short) retention and higher plasma copper levels, as evaluated by the Buschke-Fuld Selective Reminding Test, as well as poorer concentration abilities, as tested by the Blessed Information-Memory-Concentration Test. In their study, Bucossi and coworkers [29] analyzed in a meta-analysis design, data from all the serum, plasma, and CSF case-control studies published since 1983 on Alzheimer's disease patients, to gain an objective evaluation of whether systemic copper variation were associated with Alzheimer's disease. Data from 21 studies on serum copper and 5 studies on plasma copper were merged for a pooled total of 966 Alzheimer's disease patients and 831 controls which were enough to draw the conclusion that Alzheimer's disease patients had higher levels of serum copper than healthy controls, sufficient to unambiguously distinguish Alzheimer's disease patients from healthy controls. From a totally different perspective the same authors challenged the copper hypothesis in Alzheimer's disease, screening chromosomes from Alzheimer's disease patients for Wilson's disease mutations or polymorphisms [30]. They found that the Wilson's disease ATP7B gene—which is a tight control balance regulator for free copper levels in the body—presents susceptibility loci for late-onset Alzheimer's disease [30]. As stated by the authors, they explored Alzheimer's disease chromosomes with a hypothesis-driven candidate gene association study, to verify whether the Wilson's disease ATP7B gene had susceptibility loci for late-onset Alzheimer's disease, assuming that the free copper disproportion is true and specifically associated with AD. Moreover, the authors gained further results on an additional exon 12 single nucleotide polymorphism (SNP) of the ATP7B gene associated with Alzheimer's disease and in linkage disequilibrium with the exon 10 SNP one, suggesting that exon 12 or something very close to it can be a susceptibility locus for Alzheimer's disease (R. Squitti personal communication). It would be surprising that genome-wide association studies (GWASs) carried out so far have never found an association between Alzheimer's disease and the 13q14.3 DNA region where the ATP7B gene lies. However, to this regard, another paradigm shift recently proposed should to be considered [36]. In fact, current experience with GWASs suggests that rarer variants that are, actually, hard to detect by GWASs, may account for the missing hereditability of Alzheimer's disease, estimated around 58–79%, and which plays a role in the development and progression of Alzheimer's disease [37]. The paradigm recently proposed of a shift from the "common disease—common variant hypothesis "to a "common disease—multiple rare variants hypothesis" may suitably fit to the ATP7B gene association with Alzheimer's disease, since the gene is highly polymorphic but at the same time harbors rare mutations, that in homozygous or heterozygous compounds trait are causative of Wilson's disease (Squitti R. personal communication). In other words, it seems not premature to sustain the hypothesis that type 2 free copper toxicosis may play a causal role in age related Alzheimer's dementia.

Glossary

Paradigm Shift. a radical change in thinking from an accepted point of view to a new one. For instance, as to neurodegeneration: from no causal treatment available to zinc therapy for treatment of free copper toxicosis.

Deductive Method. This evidence based method aims at identifying errors and fallacies. Randomized clinical trials are based on the deductive method.

Evidence Based Medicine. The conscientious use of current best deductive evidence in making decisions about the care of individual patients.

Free Copper. The small portion of plasma copper that is not bound to ceruloplasmin. Free plasma copper is the toxic form of the metal. Copper accumulated in deposits in Alzheimer plaques is not toxic.

Inductive Method. This method aims at verification of the theory but not at detecting its errors. It may lead to entrapment in fallacies.

Penicillamine Fallacy. The tragic, inductive method based misconception that treatment of copper toxicosis can best be started with a chelating agent like penicillamine.

Seductive Method. The unscientific method of choosing a therapy simply on the basis of expert opinion, pharmaceutical representatives or advertisements.

Type 1 (Juvenile) Free Copper Toxicosis in Wilson's Disease. The type of free copper toxicosis causing oxidative stress with free radicals leading to neurodegeneration especially in the basal ganglia.

Type 2 (Age Related, Senile) Free Copper Toxicosis in Alzheimer's Disease. Conscientious analytic cumulating of the available evidence has led to the hypothesis that free copper toxicosis does catalyze formation of amyloid in plaques and oxidative stress causing neurodegeneration in Alzheimer's disease.

Zinc Therapy. A randomized clinical trial testing a clear hypothesis is needed before conclusions can be drawn about the value of zinc supplements in the treatment of Alzheimer's disease. Such a clinical trial could be set up on lines similar to the randomized clinical trial of penicillamine performed in 2002 [25]. The effect of a low dose of zinc (50 mg/day) on the free copper concentration in serum, the urinary excretion of copper, laboratory markers of oxidative stress, and cognitive functions could be investigated in a blind, placebo-controlled trial. In conclusion, the work of Squitti et al. has provided data to justify the hypothesis that free copper concentrations are raised in patients with Alzheimer's disease. In my opinion, it is justified, and even desirable, to test in a controlled study the hypothesis that the free copper poisoning of patients with Alzheimer's disease is amenable to zinc therapy. A previous study suggested potential benefit of a zinc therapy in AD [28]. Moreover, a very preliminary study with Zinc therapy gave positive results in counteracting AD progression [28]. Specifically, a previous study suggested potential benefit of a zinc therapy in AD [28]. Ten patients were treated, all of them receiving 50 mg of oral Zinc bis-(DLhydrogenaspartate) three times daily obtaining improvement of memory, understanding, communication, and social contact in eight patients, as stated by the author [28]. The discontinuation of the treatment decreased and even reversed the improvement, in all patients. However, these conclusions have to be taken very cautiously. Even though these limitations, a phase II, multicentre, prospective, randomized, double-blind, placebo-controlled, parallel group study conducted with patients presenting a diagnosis of mild to moderate clinical trial with the following characteristics: (a) a treatment duration of at least one full year, as expected with curative compound use versus symptomatic approaches; (b) a patient-inclusion criterion based on individual serum Cu-dysfunction evidence; (c) monitoring of Cu bioavailability throughout the study with detection of Cu metabolism markers such as free Cu, ceruloplasmin, or Cu/Zn superoxide dismutase levels, to prevent adverse events; (d) statistical power (at least 75 patients per arm, placebo and treated); a dose of Zinc Sulphate 400–1200 mg/day, can be proposed.

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Review Article

Issues Raised Involving the Copper Hypotheses in the Causation of Alzheimer's Disease

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I present evidence that the epidemic of Alzheimer's disease is a new phenomenon exploding in the latter part of the 20th century in developed countries. I postulate that a major causative factor in the epidemic is the coincident use of copper plumbing, and the ingestion of inorganic copper leaching from the copper plumbing. I present evidence to support this hypothesis and discuss various objections and criticisms that have been raised about the hypothesis, and my responses to these criticisms. I conclude that the hypothesis is well supported by the evidence and deserves serious consideration, because if it is valid, it indentifies a partially preventable cause of Alzheimer's disease.

1. Introduction

Alzheimer's disease (AD), and mild cognitive impairment (MCI) that precedes it, has become a huge medical problem. The numbers are staggering. In the US there are currently about 5 million cases of AD and a similar number of cases of MCI [1]. We are in the US, so we are most familiar with US numbers, but there is a similar high incidence in Europe and most other developed countries of the world. The economic costs are also staggering. These patients live many years after the diagnosis and require increasing health and caregiver care. Perhaps most important, the affected patients are robbed of a reasonable quality of life during their elderly years. As the disease progresses, they gradually become unaware of what is going on around them, fail to recognize their loved ones, and lose the ability to function. This loss of quality of life and dignity in such a large proportion of our elderly should be unacceptable to us as a society if it is at all preventable. We should leave no stone unturned to find preventable causes, if any exist, and to develop new therapies.

We have come up with a hypothesis for a plausible, potentially preventable, cause, namely, ingestion of inorganic copper [2]. We do not claim this as a sole cause, but rather it, along with a high fat diet, sets the stage for the disease to develop, particularly if other risk factors are present.

What do we mean by inorganic copper? We differentiate what we call organic copper, which is copper in food, and which is bound up in organic protein molecules, from simple salts of copper not bound to anything. The latter, inorganic copper, is the kind of copper present in drinking water and in copper supplements present in most available vitamin/mineral supplement pills. Since there is almost no protein in uncontaminated drinking water, copper leached off copper plumbing pipes will combine with the cations found in drinking water, such as sulfates, carbonates, and phosphates, to form copper sulfate, copper carbonate, and copper phosphate. Copper added to vitamin/mineral supplement pills is also a simple salt, often copper sulfate. As I will discuss, I believe inorganic copper, at least in part, is handled differently by the body than organic copper, and ends up in different places.

At this point, I will briefly review some aspects of the metallochemistry of AD related to copper and zinc, so that less specialized readers may have a better understanding of the points being discussed. One of the hallmarks of AD pathology in the brain is extracellular amyloid plaques [3]. These are polymers of beta amyloid, a polypeptide clipped

off the end of the amyloid precursor protein. The amyloid plaques are thought to be toxic to neurons and are thought to be an integral part of the pathogenesis of AD. These plaques are very rich in copper and zinc, and for a time it was thought both elements were involved in plaque formation [4, 5]. However, it now seems likely that copper is required for plaque formation while zinc is simply bound in large amounts to the plaques. Indeed the plaques, representing a sink for zinc, may add harmful depletion of zinc in the brain [6]. It is already known, based on serum zinc levels, that AD patients are zinc deficient [7], and zinc plays important roles in neuronal function. Thus, copper may be incriminated as a toxic agent, while zinc may be protective, and the brain zinc deficient. Copper also contributes to generation of toxic oxygen radicals in interaction with amyloid plaques [8]. These roles make extracellular copper a potential culprit in the pathogenesis of AD.

Adding further fuel to the fire of a role for copper in AD pathogenesis is a series of papers from an Italian group led by Dr. Rosanne Squitti. This group has focused on "free copper" in the blood. Free copper is the copper in the blood not covalently bound to ceruloplasmin, a copper containing protein in the blood that accounts for 60–65% of blood copper. The remaining copper is called free copper, although it is not really free, but is loosely bound to molecules such as serum albumin. Although not technically "free", this copper is freely available to contribute to cellular needs, and if the free copper pool is expanded, to cause toxicity. This is most markedly seen in untreated Wilson's disease, an inherited disease of copper accumulation and copper toxicity [9]. The greatly expanded free copper pool in this disease is associated with copper toxicity in brain and liver. Squitti and colleagues have found that the free copper pool in the blood is also increased in AD patients [10], that the increase in free copper is correlated with a cognitive measure [11] and is predictive of cognitive decline over the next year [12]. Although these data do not prove copper causation, they are strong pieces of evidence consistent with the copper causation theory.

In 2003, Sparks and Schreurs [13] did a landmark study that strongly indicated that trace amounts of copper in drinking water are toxic to the brain. They showed that addition of 0.12 ppm copper to the drinking water of a rabbit model of AD greatly enhanced the amyloid plaque pathology in the rabbit brain, but also caused a strong deterioration in cognition. This work has been replicated in other AD models [14], and by other workers [15]. For reference, the EPA allows 1.3 ppm copper in US drinking water for humans, over 10-times the level enhancing AD in the animal models. For further reference, if this amount of copper were increased in food, it would have a trivial, nontoxic effect. The human diet ranges from about 0.8 mg to 1.4 mg/day of copper content. 0.12 ppm additional copper would add 0.12 mg/day, about 10% of the copper already there, and much less than the 50% plus variation in copper already present in the diets. These data indicate that copper from drinking water is much more toxic to the brain than copper from food. I differentiate these two types of copper by referring to copper in drinking water as inorganic copper, as opposed to the copper in food, which is bound to food protein, which I call organic copper. Since there are no significant amounts of protein in uncontaminated drinking water, by the above definition copper in drinking water has to be inorganic.

Although not specifically focused on AD, there is another study which speaks to the potential brain toxicity of inorganic copper. Morris and colleagues [16] did a large study in Chicago in which they evaluated intake of various nutrients and looked at cognition change over a several-year period. They found that subjects in the highest quintile of copper intake, if they also ate a high fat diet, lost cognition at six-times the rate of other groups. Subjects were in the highest quintile of copper intake by virtue of daily ingestion of vitamin-mineral supplement pills containing 1–3 mg of copper. This copper is inorganic by our definition, because there is no protein in these supplement pills.

In a very nice review of whether there is too much or too little copper in Alzheimer's disease, Quinn et al. [17] reviews the evidence for both sides. He keeps an open mind but seems to favor two clinical trial approaches. One is copper lowering, with agents like tetrathiomolybdate or zinc, using ceruloplasmin levels as a guide to copper status. The second is a copper redistribution approach in which the drug, such as one called PBT2, may redistribute copper from extracellular to intracellular compartments. This approach envisions an intracellular copper deficiency being present.

Returning to our inorganic copper causation hypothesis for AD, as we have put this hypothesis forward in various publications, presentations, and manuscript submissions, we have run into a blizzard of objections, counter arguments, and denial. These have come from publications, from reviewers of our papers, from communications with workers in the field, and Alzheimer's physicians. While we expect a good deal of skepticism because our ideas about inorganic copper as partially causative are quite different from what has been generally believed, we are a bit taken back by some of the arguments against our hypothesis being definitely and provably wrong, and others more or less obviously wrong. In some cases there is obvious conflict of interest, with objections coming from the copper industry and those funded by the copper industry. In other cases there is probably bias against a new idea because people have become comfortable with their own existing ideas. In other cases there may be discomfort with recognizing a major harmful factor in our environment that perhaps should have been recognized some time ago, if not long ago.

Thus, I have put this paper together to list the set of subhypotheses that lead to our overarching hypothesis, and to discuss the issues that have been raised surrounding each. I acknowledge the fact that ingestion of inorganic copper as a partially causative factor in AD is still an incompletely proven hypothesis, but I point out there is compelling evidence to support it. So, while my concepts are still an unproven theory, I hope our skeptics will keep an open mind and examine the evidence. Even if someone views our theories as only having a slight chance of being correct, if they do prove correct, it is extremely important for AD prevention. So let us get on with the debate, and the further work needed to see if we can eliminate a preventable cause of AD.

2. The Copper Causation Hypothesis

Overarching Hypothesis. Ingestion of inorganic copper from drinking water and from copper supplements is a risk factor for AD and a major factor causing the AD epidemic.

Subhypotheses

(1) The epidemic of AD is a new disease phenomenon and is associated with development.

(2) Ingestion of inorganic copper leached from copper plumbing is a major factor in causing the AD epidemic, with copper supplements possibly playing a subsidiary role.

(3) Ingestion of inorganic copper contributes directly to the free copper pool in the blood.

(4) Elevated free copper in the blood of AD patients is a pathogenic factor in AD.

(1) Subhypothesis 1: The Epidemic of AD Is a New Disease Phenomenon and Is Associated with Development

The relationship of this subhypothesis to copper is that in subhypothesis 2 I am going to postulate that the epidemic of AD is associated with the explosive use of copper plumbing in the 20th century, and that copper leaching from the copper pipes is partially causal of AD. Thus, I wish to establish that the epidemic of AD represents something new, not present, or hardly present, in the 19th century, for example, and is associated with use of copper plumbing in the 20th century.

Supporting Data. Regarding the question of whether the AD epidemic is a new disease phenomenon, the book by Waldman and Lamb [18] is very well researched on this topic. As far as I know, these are the first authors to suggest the AD epidemic is a new disease phenomenon, and if so, deserve credit for this concept. They reviewed the extensive writings of disease authorities who published in the latter part of the 19th century, and found, consistently, that AD did not exist, or if it did, it must have been rare. There is the writing of Osler, an internist, who compiled a seven-volume series of books, with the help of colleagues, on all medical diseases. Volume 7, almost 1000 pages long [19], was on diseases of the brain and nervous system. There is no mention of an AD-like disease. Similarly, Gowers, a neurologist, wrote extensively on brain and neurologic diseases, and did not mention an AD-like disease [20]. Similarly Freud (work collected by Strachey et al.) who wrote extensively on psychiatric disease, did not describe an AD-like disease [21]. Finally Boyd, who wrote a comprehensive textbook on pathology, which was put out several times as updated new editions, did not describe the hallmarks of AD pathology in the brain, amyloid plaques, and neurofibrillary tangles [22].

Since AD is a disease of aging, Waldman and Lamb [18] wondered whether lack of elderly people in the population when Osler, Gower, Freud, and Boyd made their observations was the cause of the absence of the disease in their writings. To the contrary, they found that there was always a substantial fraction of people over 60 in the relevant populations. For example, in France, in 1911, more than half of the population was still alive at age 60.

I have examined the US census figures for 1900 and found that there were 3,184,363 people over age 60. At today's prevalence, there should have been over 36,000 US cases, more than enough for Boyd to find positive AD brains at autopsy.

Regarding the question of whether the AD epidemic is largely associated with development, Ferri et al. [23] give a summary of data for developed and undeveloped countries. In general the data show a considerably higher frequency of AD in developed countries.

Contrary Data, Contrary View, and My Responses:

Contrary View. A common view among AD workers is that AD was always present, but the dementia was simply passed over as part of normal aging, and no special attention was paid to these patients.

My Response. This might account for the lack of attention by the clinicians (Osler [19], Gowers [20], and Freud [21]), although it is a little hard to believe given their quality and attention to detail, but it does not explain why the pathologist Boyd [22] did not see amyloid plaques and neurofibrillary tangles, pathological hallmarks of AD, in the brains of patients autopsied.

Contrary View. Another common view is that the AD incidence is increasing only because the disease is now better recognized and labeled, and because of the increasing number of elderly people in the population.

My Response. I agree that both of these factors are involved in explaining the increasing prevalence. However, my position is that there has to be something additional to explain the absence of brains with AD pathology around the beginning of the 20th century [22], and the very high current frequency of such brains at autopsy. We pointed out in the previous section that an elderly population was present in France and the US 100 years ago, so simply more aged people in the current population cannot explain the almost total absence of the disease back then.

Contrary View. Another view is that the high fat, high calorie diets of developed countries are a major causative factor and a major reason for the explosive epidemic of AD in the Western world. Indeed, Grant [24] has shown a strong positive correlation between fat intake and AD prevalence across numerous countries.

My Response. That a high fat diet is a major causative factor for AD is a reasonable hypothesis. My hypotheses is that a high fat diet interacts with copper to contribute to causation. Thus, the Sparks and Schreurs [13] study involved cholesterol feeding in the rabbit model where trace amounts of copper greatly enhanced the disease, and the Morris et al. [16] study found that those in the highest quintile of copper intake, also eating a high fat diet, had the greatest decline

in cognition. There are biochemical reasons why the two factors might interact in causation, for example, copper oxidizes cholesterol and other fats into substance toxic to the brain. However, a major role for copper whether in the presence of a high fat diet or not, is supported by trace copper enhancement of AD-like disease, in the mouse model of AD, which does not receive a fat- or cholesterol-enriched diet [14, 15]. Thus, my current view is that both factors are causative, and interact together to further enhance causation.

(2) Subhypothesis 2: Ingestion of Inorganic Copper Leached from Copper Plumbing Is a Major Factor in Causing the AD Epidemic with Copper Supplements Possibly Playing a Subsidiary Role

Supporting Data. To give the reader insight as to possible involvement of copper in AD causation, I would like to briefly review the interaction of copper with the molecules intimately involved in AD pathogenesis. Beta amyloid, the key molecule in amyloid plaques, binds copper and cholesterol, causing oxidation of cholesterol to 7-OH cholesterol, extremely toxic to neurons [25, 26]. Beta amyloid is cleaved from amyloid precursor protein by beta secretase. Amyloid precursor protein binds copper, and reduces it from copper (II) to copper (I), which produces oxidative damage [27, 28]. Beta secretase also binds copper for activity. Both amyloid plaques and neurofibrillary tangles, hallmarks of AD brain pathology, are major sites of generation of toxic oxygen radicals [29]. Deferoxamine or EDTA, an iron or general metal chelator, respectively, abolishes the generation of these radicals, but replenishment with iron or copper restores the activity [29]. Tau protein, a major building block for neurofibrillary tangles, binds copper, which is a causative factor in tau aggregation [30].

In response to the six contrary views later in this section, I will show how AD risk factors, apolipoprotein E4, homocysteine, levels, and certain transferrin alleles, all tie in with the copper causation hypothesis.

A major initiating stimulus for all of our thinking and leading to the hypotheses proposed here was the 2003 study of a cholesterol-fed rabbit model of AD by Sparks and Schreurs [13]. By accident, but also by serendipity, they discovered that trace amounts of copper (0.12 ppm) added to the otherwise distilled drinking water of these rabbits, greatly enhanced the AD-like pathology in the brains, and greatly decreased the rabbits' ability to perform previously learned tasks. In other words, trace amounts of copper in the drinking water caused the two hallmarks of AD, amyloid plaques and loss of cognition, to worsen. For reference, the US Environmental Protection Agency (EPA) allows up to 1.3 ppm copper in human drinking water, over 10-times as much as worsened AD in the rabbit model. Similar and even higher, levels of copper are permitted in drinking water around the world. Sparks et al. [14] later confirmed the AD enhancing effects of trace amounts of copper in noncholesterol-fed AD models, such as in the mouse model. The effect of these very low amounts of copper added to drinking water enhancing AD pathology was confirmed by a group from Rochester [15].

Table 1: Copper levels in Nor American household drinking water.

Copper level (ppm)	Number of households		
Undetectable	80 (28%)	12	Considered safe
0.01		68	
0.02	114 (41%)	36	Safety unknown
0.03		16	
0.04		13	
0.05		15	
0.06		12	
0.07		7	
0.08		7	
0.09		8	
0.1	81(29%)	3	Cause Alzheimer's disease in animal models
0.2		34	
0.3		14	
0.4		9	
0.5		6	
0.6		2	
0.7		3	
0.8		2	
0.9		2	
1.0		2	
1.1		2	
0.2		1	
1.3		1	
1.4	5 (1.8%)	1	Above EPA limit
1.8		1	
1.9		1	
3.4		1	
6.0		1	

There is a rough correlation between the use of copper plumbing tube around the world and the incidence of AD. This is consistent with the hypothesis that copper leaching from copper plumbing is causing AD.

Are there any data on whether significant amounts of copper are actually present in the drinking water of a developed country, such as the U.S? Fortunately, there is. As a result of evaluating copper levels in drinking water of my Wilson's disease patients, I accumulated data on 280 samples of home drinking water from households all across North America. The data are shown in Table 1. The data show that 2% of samples are over the EPA limit of 1.3 ppm, 31% are over 0.12 ppm, the level that enhanced AD in the rabbit and other animal models, 41% are between 0.12 ppm and 0.01 ppm, a level of unknown toxicity/safety, and only 28%

are less than 0.01 ppm, a level we consider safe. Thus, 72% of these North American drinking water samples either have copper levels above that enhancing AD in animal models, or are high enough to be of unknown safety in that regard.

The other common source of inorganic copper ingested by humans is copper in vitamin/mineral supplements. In the last 50 years or so it has become very common in the US, and probably many other developed countries to ingest one or more of these supplement pills per day. Almost all of these pills have 1 to 3 mg of copper. Since the dietary intake of copper is about 1.0 mg per day [31–34], this is a hefty dose of copper, and it is all inorganic. Is there any evidence that this high dose of inorganic copper has an effect on cognition? Yes, in a large study of a general Chicago population published in 2006, Morris and colleagues [16] found that those in the highest quintile of copper intake, if they also ate a high fat diet, lost cognition at six-times the rate of the rest of the people. These people were in the highest quintile of copper intake because they took mineral supplement pills containing copper.

Contrary Data, Contrary Views, and My Responses. Recently I wrote a letter to the editor criticizing a collection of five papers published in one issue of the Journal of Toxicology and Environmental Health [35]. These papers concerned the safety and toxicity of oral copper intake. In general, these papers were nice reviews of various aspects of the topic. My two main criticisms in my letter to the editor were that none of the five papers differentiated the toxicity of inorganic copper in drinking water or in copper supplements from the toxicity of copper bound to organic molecules in food, and none of them referred to the literature emerging since 2003 on the toxicity of this inorganic copper. There was a lengthy response senior authored by Danzeisen et al. [36] to my letter by 8 of the 14 authors of these five papers plus four other scientists who were recruited to join in on the discussion. In spite of the length of this response, it did not address the two main criticisms listed above. But it did criticize many aspects of the data cited in my letter that pointed to the potential toxicity of inorganic copper ingestion. These criticisms as they relate to the inorganic copper causation of AD hypothesis will be discussed below.

Contrary View. One of the criticisms in the Danzeisen et al. [36] paper was leveled at the Sparks and Schreurs [13] rabbit model study. The authors mistakenly asserted that the study only showed amyloid plaque reduction in the animals, and that plaque reduction may not be related to "progression of dementia."

My Response. Danzeisen et al. [36] are mistaken. The Sparks and Schreurs [13] paper showed not only plaque reduction but loss of cognition in the copper treated animals.

Contrary View. The Danzeisen et al. [36] paper criticizes Brewer et al.'s [35] conclusion from the Sparks and Schreurs [13] study that copper exposure from drinking water in itself can be a risk factor for AD. Danzeisen et al. [36] are concerned about drawing conclusions from a rabbit model of AD in which high cholesterol feeding preconditions the animal to AD, so that worsening of the disease from addition of copper to drinking water cannot be used to conclude that copper alone in drinking water is a risk factor for AD. To quote them, "This type of study in compromised animals is applicable to neither the derivation of the maximum contaminant level (MCL) for copper in drinking water, nor its extrapolation to the nonobserved-adverse-effect level (NOAEL) for copper in humans."

My Response. In my opinion drinking water should be nontoxic and safe for all segments of the population. When I served on the Committee to Evaluate Copper in Drinking Water convened by the National Research Council in 2000 [37], we considered, for example, whether the current allowable level of copper was safe for Wilson's disease heterozygous carriers, who have minor extra accumulation of copper, and comprise only 1% of the population. Similarly, if the segment of the population who eat a high cholesterol, high fat diet, which is increasingly common in our society, are especially vulnerable to get AD if they ingest low levels of copper in drinking water, we must try to make the water safe for them. And I point out that the mouse AD model, which also showed vulnerability to 0.12 ppm copper in drinking water, does not depend on cholesterol feeding to produce the disease [14].

I believe we have to be more comprehensive in assessing the safe limits of copper in drinking water. The standard methods consist of assessing acute effects of copper levels in drinking water in humans, but chronic effects of copper toxicity are evaluated using animal studies of copper in food. The latter is inadequate, because the evidence seems pretty clear from the AD animal model work that copper is much more toxic in water than it is in food. If it takes special animal model studies to reveal potential vulnerability of humans to copper in drinking water for a specific disease, we should pay attention to those studies, not ignore them by saying the animals were "compromised" to produce the model.

Contrary View. Another one of the criticisms in the Danzeisen et al. [36] paper was the Brewer et al. [35] citation of the work of Morris et al. [16] referred to earlier. In this work Morris et al. [16] found that those in the highest quintile of copper uptake, most being in this quintile because of taking copper supplements, if they also ate a high fat diet, suffered cognition loss at six-times the rate of other groups, Danzeisen et al. [36] state, "It is not possible to separate the factors studied by these authors and implicate copper directly in the presence of high fat intake." Danzeisen et al. [36] also worried about separating the effect of copper intake from the other ingredients of vitamin/mineral supplements.

My Response. Regarding the criticism of copper being toxic only in the presence of a high fat intake, we point out that these were normal people eating their normal diet. Surely those eating this kind of diet which is an increasing number of our people, also need protection from toxicity resulting from ingestion of inorganic copper in supplement pills that singles them out for cognition loss. The FDA, needs to

evaluate the potential toxicity of copper in supplements. Regarding sorting out the effects of copper versus other ingredients in the supplements, Morris et al. [16] did that in their statistical analysis. Copper, plus a high fat diet, were the only culprits.

Contrary View. Danzeisen et al. [36] contradict the Brewer et al. [35] statement that, "No formal toxicology studies of copper in drinking water have ever been done," by directly saying this "is not correct."

My Response. Danzeisen et al. [36] never specifically refute the Brewer et al. [35] statement. Instead they cite various types of data which are not relevant to the point, including an assessment, not based on a chronic toxicity study of copper in drinking water, by the European Union, that up to 2.0 mg/L (2.0 ppm) in water is safe, with the average intake currently being 0.7 ppm.

Contrary View. Danzeisen et al [36] cite studies by Kessler et al. [38] in which AD patients were given 8 mg of copper as copper orotate/day for one year. The cognition of the patients did not get worse, leading to the conclusion that long-term oral intake of copper is not a risk factor for AD.

My Response. These patients did not improve cognition either! The hypothesis underlying the study was that AD patients were copper deficient, and copper therapy would be efficacious. Thus, the study was a negative one in terms of its underlying hypothesis. However, because the patients did not get worse, the authors turned their conclusion around, and concluded copper administration was not harmful.

As to why the patients did not get worse given our hypothesis about the role of copper toxicity, there are problems with this study leading to many possible explanations. Copper orotate is relatively insoluble, and perhaps very little was absorbed. No copper parameters were measured in the patients, so there is no information on absorption. It is possible that maximum effects from exogenous copper were already occurring, and that more exogenous copper therefore had no additional effect. Finally, AD is a slowly progressive disease, and these were mild patients. Perhaps the observation period was not long enough to affect the cognitive tests being carried out.

Contrary View. Danzeisen et al. [36] criticize Brewer et al.'s hypothesis that copper plumbing is a causal factor in AD. They say genetic factors play a "particularly important role" in AD, with age being the greatest nongenetic risk factor. They say "we are not aware of any peer-reviewed scientific evidence that supports Brewer et al.'s [35] conjecture that the use of copper piping is a risk factor in AD."

My Response. I agree that there are genetic risk factors such as the apolipoprotein E4 alleles [39] and certain genes involved in controlling iron, such as certain hemochromatosis alleles [40] and certain transferrin alleles [41]. In addition, elevated homocysteine levels are a risk factor [42]. Interestingly, these all tie in with our hypothesis. The apo E4 protein lacks a copper binding cysteine, so it cannot bind copper while the apo E3 and E2 alleles have this cysteine. Thus apo E4 is unable to remove copper from the brain. Homocysteine interacts with copper to oxidize cholesterol, damaging to neurons [43]. Iron build up increases oxidant stress, as does copper build up, so the two work in the same manner. It is our view that copper toxicity and a high fat diet [24] set the stage for AD to develop, and other risk factors operate on that stage to increase the likelihood of AD to develop. Regarding the comment about no peer-reviewed evidence (in other words no published paper) exists to support our hypothesis about copper plumbing as a risk factor for AD, the explanation is that we claim priority as the first to propose this hypothesis. There has to be a first worker to propose a new hypothesis. If the hypothesis is correct, supporting papers will come later.

Contrary View. Danzeisen et al. [36] cite a paper by Crouch et al. [44] which presents evidence that there is an intracellular copper deficiency in the brain of an AD mouse model. They interpret this to mean that ingestion of additional inorganic copper would be a helpful rather than harmful. They also cite papers which they say supports the idea of an overall low level of copper in the human AD brain.

My Response. I do not agree that it is clear that the AD brain has a low copper value. There is just as many papers that find copper levels elevated or normal in the AD brain as find it low. So this area is controversial. However, the paper by Crouch et al. [44] is interesting. These authors administer a copper binding agent to AD model mice. The agent is designed to release its copper in the intracellular environment. This agent improved AD-type pathology and improved cognitive performance in a mouse model. A sister compound which bound copper but did not release it intracellularly had no effect. Similar claims for the drug clioquinol are not as clear, because it is not certain that clioquinol releases bound copper intracellularly, and clioquinol also binds and transports zinc.

The data in the Crouch et al. [44] paper do suggest that increasing the intracellular copper levels in the mouse AD model is beneficial. This possibly indicates that there is intracellular copper deficiency. However, there appears to be overlapping functions of copper and zinc in the synapse, and recently it has become clear that AD patients are zinc deficient [7]. It is possible that the effect of copper in the Crouch et al. [44] study is substituting for a relative lack of intracellular zinc.

However, even if an intracellular copper deficiency exists, there is also evidence that extracellular copper enhances oxidant damage in interaction with amyloid plaques and A beta amyloid oligomers. If there is an intracellular copper (and/or zinc) deficiency, and an extracellular copper toxicity, then therapy can be aimed at both, restoring intracellular copper (and/or zinc) levels, and reducing extracellular copper levels. If intracellular copper deficiency exists, it does not negate our hypothesis that excess ingestion of inorganic copper contributes to AD development because of extracellular copper toxicity.

Contrary View. It has been suggested that the source water, rather than copper plumbing, may contribute the toxic copper to drinking water.

My Response. This may be true in parts of the world, and in a few areas of the US. But in many areas of the US, source water has low copper.

Contrary View. The epidemiologic data showing a low rate of AD in Japan [45], a developed country, that shuns copper plumbing, which we use to support the copper plumbing causal hypothesis, has been criticized, because Japanese have a diet different than other countries. We have pointed out that when Japanese migrate to Hawaii, where copper plumbing is common, they develop a rate of AD typical of developed countries [46], in further support of our hypothesis. This concept has been criticized because the diet and other environmental factors may also be different in Hawaii.

My Response. We agree the epidemogic data, including the Japanese epidemiologic data, do not prove our hypothesis. The data, including the somewhat unusual Japanese data, simply show consistency with the hypothesis.

Subhypothesis 3: Ingestion of Inorganic Copper Contributes Directly to the Free Copper Pool in the Blood

Supporting Data. Normally food copper is metabolized by the liver and channeled into safe pathways such as being incorporated in to ceruloplasmin. The exceptional toxicity of trace amounts of inorganic copper in drinking water in AD animal model studies [13–15] suggests that at least some of this copper must follow a different route. We do have a little direct data that this is indeed true. When we administer inorganic copper orally labeled with ^{64}Cu, we see a fraction of the label appear in the blood in 1-2 hours, too soon to be processed by the liver [47]. The amount appearing is 5-6% of the administered label at both the 1- and 2-hour time points, so a guesstimate of the area under the curve, a rough estimate of absorption, is about 15%. We believe this fraction of ^{64}Cu-labeled inorganic copper is bypassing the liver, is therefore not incorporated safely into ceruloplasmin, contributes directly to the nonceruloplasmin free copper pool, and represents what is happening to at least a portion of the inorganic copper ingested in drinking water or copper supplements. We hypothesize that this pathway for ingested inorganic copper is a causal factor in AD.

Contrary View. One criticism of the above is that Ctr 1 is the major copper transporter in intestinal cells [48], so it does not seem likely that inorganic copper can bypass the liver and enter the free copper serum pool directly.

My Response. The animal model AD data [13–15] indicating the toxicity of trace amounts of inorganic copper in drinking water, and our ^{64}Cu data [47], indicates something different is happening with at least a portion of ingested inorganic copper. However, I agree that the mechanism by which some of the inorganic copper is differently absorbed is unknown. We can speculate that Ctr 1 may only recognize organically bound copper, and some inorganic copper is absorbed through different channels, such as cation channels. Also, it is possible that inorganic copper is absorbed quickly, as a bolus, and some simply bypasses the liver because of the high level. At this point the mechanism is unknown.

Contrary View. Danzeisen et al. [36] criticize our ^{64}Cu data by saying that we do not know how much inorganic copper actually bypassed the liver and promptly appeared in the blood because the specific activity of the radioactive copper was not measured. They also say, since it was given with milk, some of it probably became protein bound.

My Response. Regarding the first criticism, it represents a misunderstanding of this kind of study. It is not necessary to know the specific activity to know the proportion of the label that was involved, which is what is of interest. Regarding the second comment, some of the label probably did bind to ovalbumin becoming "organic" copper. Thus, if the ^{64}Cu had been given in water and remained completely inorganic, the 1- and 2-hour blood values might have been considerably higher.

Subhypothesis 4: Elevated Free Copper in the Blood of AD Patients Is a Pathogenic Factor in AD

Supporting Data. Elegant data on this point has been published by Squitti and her colleagues [10–12, 49]. They have shown the following.

(1) Free copper in the blood is elevated in AD patients [10].
(2) Free copper negatively correlates with cognition in AD patients [11].
(3) Free copper levels predict the rate of loss of cognition in AD patients [12].

They also showed that free copper negatively correlates with cognition in older normal women [49].

Number (1) above (elevated free copper in AD) has been confirmed [50, 51]. Our groups finds a higher percentage of free copper in AD, and increased defective ceruloplasmin, that is, ceruloplasmin that has lost its enzymatic activity because it has lost some of its copper. We do not know whether this process contributes to a higher free copper. Arnal et al. [51] confirm the higher free copper in AD and also confirm a significant negative correlation between serum free copper and cognition.

Contrary View. Danzeisen et al. [36] find fault with the way Squitti et al. [10–12, 49] determined free copper "values arithmetically by measuring Cp-bound copper and subtracting the value from total copper." They say, "There is a scientific consensus (voiced by seven experts in the field in a peer-reviewed article: Danzeisen et al. [52] that non-Cp copper determined by arithmetic method is not sensitive to copper status, and that its true value is very difficult to measure".

My Response. First, non-Cp copper determined arithmetically is not "difficult to measure." We have been doing it

for years in our Wilson's disease work [9, 31, 53]. One simply determines the Cp level by either an enzymatic or immunologic method, calculates the amount of copper bound to Cp from the known amount of copper bound to each mg of Cp, and subtracts that from total serum copper. Of the two ways of measuring Cp, the enzymatic is probably a little better, because in the immunologic method, a little apo-Cp (without copper), is usually measured, the amount determined by the antibody used. But either way, if the same method is used consistently, it gives a very good estimate of the non-Cp copper and its change over time.

If this methodology is so useless, why is it so elevated in untreated Wilson's disease patients experiencing acute copper toxicity [9]? And why did it change so consistently and predictably in our Wilson's disease therapy studies [9], And, coming back to the data being criticized, why does it correlate so beautifully with cognition measures in AD [11], why does it predict cognition loss in AD [12], and why does it correlate with cognition in elderly women [49]? It is not credible to believe all of this is coincidence or random concurrence? Even the staunchest critics, even if they do not believe non-Cp copper is in the pathologic pathway for AD, have to admit it is at least a good marker of the AD process.

Contrary View. I have heard criticism that other authors in the literature have reached opposite conclusions. I have already discussed and criticized the work of the Bayer group [38] who administered copper orotate to AD patients. This same group published a paper that showed a lower total serum copper and ceruloplasmin in AD patients with cerebrospinal diagnostic markers, than AD patients without those markers, suggesting severe patients are copper deficient when compared to less severe patients [54].

My Response. This work is flawed in that total serum copper was measured not free copper, and total serum copper will simply reflect Cp levels, since Cp contributes up to 90% of serum copper.

Contrary View. Bayer et al. [55] have also published a paper showing that dietary copper supplementation reduces amyloid A beta production in an AD mouse model.

My Response. However, these mice are severely copper deficient, and copper supplementation helps them in many ways because of this.

Contrary View. Klevay [56] has written a review entitled, "Alzheimer's disease as copper deficiency." Klevay cites the work of the Bayer group [38, 54, 55] already discussed here in support of his hypothesis.

My Response. The title of this review is misguided in the sense that it ignores the evidence for what the syndrome of copper deficiency really is. For example, in recent years the syndrome of copper deficiency has been amply illustrated by excessive use of zinc containing-denture adhesive. The zinc eventually causes severe copper deficiency. These patients develop pancytopenia followed by myelopolyneuropathy, which often leaves them severely neurologically crippled [57, 58]. None of this occurs in AD, so it is not reasonable to suggest that AD is a simple copper deficiency [56].

However, as we have previously discussed, we accept the possibility that AD patients have a neuronal intracellular copper deficiency. But this is very different than what Klevay [56] and the Bayer group [38, 54, 55] have been proposing.

Contrary View. An opinion has been expressed that the biliary copper export pathway (the one that is defective in Wilson's disease) will protect against increases in serum free copper.

My Response. My view is that this pathway is protective, but not immediately sensitive to mild increases in free copper. Thus, if the free copper levels are increased by increased intake, the export pathway will respond and increase export, but steady state levels of free copper are probably increased.

3. My Overview of the Status of These Issues

It seems to me that subhypothesis 1 is not all that controversial. Rather the doubters are just not informed. I urge them to read Dying for a Hamburger by Waldman and Lamb [18]. This is an extremely well-researched book on the topic of the AD epidemic being new, and that elderly people have always existed in our population. Even if one thinks AD was passed off as simply the normal dementia of aging, it is very difficult to explain the absence of amyloid plaques and neurofibrillary tangles in the autopsy specimens of Boyd [22]. I am not saying AD did not exist at all in the 1900s. It probably did. Probably occasionally a constellation of risk factors would come together and produce the disease. What I am saying is it was relatively rare, and that there were enough elderly people present for it to have easily shown up in the autopsy specimens if it were occurring at today's rate. Those who wish to counter this argument would have to either show that there were very few people over age 60, or that the plaques and tangles were actually present in autopsy samples.

There is more controversy about subhypothesis 2, as is evident from the length of the contrary view/My response material regarding that subhypothesis.

One area where a legitimate difference of opinion can exist is whether the Sparks and Schreurs [13] and other AD animal work [14, 15], in which trace amounts (0.12 ppm) of copper in drinking water greatly enhances AD pathology and cognition loss, can be extrapolated to the human. There are four animal models studied, one of them does not involve cholesterol feeding (the mouse), and they are all consistent. Plus the mouse work has been confirmed in another laboratory [15]. So the animal model work is very solid that trace amounts of copper in drinking water are sharply more toxic than copper in food in AD-like causation. But still, they cannot be extrapolated to the human with absolute certainty. My position is that they should be taken seriously as signaling a major potential (and preventable) cause of human AD. Certainly not ignored, as did the five papers in the Journal of Toxicology and Environmental Health [35], and not inaccurately criticised [36].

A legitimate point is that besides the increase of copper plumbing in developed countries, many other environmental changes have occurred, as well as behavioral changes in people. Thus, assuming subhypothesis 1 is correct, any of these factors could be responsible for the AD epidemic. Waldman and Lamb [18], who wrote Dying for a Hamburger, in their otherwise well-researched book previously referred to, postulated that it was beef eating, with AD caused by prions in the beef. Beef eating certainly correlates with development, but there is no evidence AD is a prion disease. However, a factor associated with development is eating more fat in the diet (including that in beef). Grant [24] has shown there is a good correlation between a country's dietary fat consumption and the prevalence of AD. Since much of the animal model data (plus the Morris et al. [16] data) involves feeding cholesterol (or ingesting a high fat diet), we believe copper and cholesterol/fat are synergistic in AD causation. Copper can oxidize cholesterol and certain fats into substances that are toxic in the brain. However, one could postulate that a high fat diet, without copper involvement, is the causative factor. This is a reasonable hypothesis. But of course it ignores all the data involving copper. Other unknown causatives factors associated with development are also possible.

I do not believe the issue of whether adequate testing has been done on the chronic toxicity of copper in drinking water is very controversial. It just has not been done. None of the statements or citations by Danzeisen et al. [36] show that this type of work has ever been done. Besides animal studies, epidemiologic studies on humans could be done, relating disease incidence to levels of substances like copper in drinking water. It is not an easy study, given that many people move around and their source of drinking water varies from time to time.

The possibility of a neuronal intracellular copper deficiency in AD, irrespective of potential toxicity from excess extracellular copper, is a viable hypothesis and supported by some emerging data [44]. This would make rational the use of therapies which deliver copper intracellularly while not adding extracellular copper. However, in the face of all the evidence of extracellular copper toxicity in AD, intracellular copper deficiency does not make rational the use of copper supplementation to AD patients as done by the Bayer group [38]. Nor does it justify a review, such as that of Klevay [56], entitled, "Alzheimer's disease as copper deficiency."

I do not believe there is much controversy about whether some of the ingested inorganic copper contributes directly to the free copper pool, as proposed in subhypothesis 3. Even Danzeisen et al. [36] referring to my interpretation of our ^{64}Cu data as showing that some ingested inorganic copper had bypassed the liver said, "this interpretation appears correct." Of course, the mechanism by which this occurs is still unknown.

Regarding subhypothesis 4, the elegant work by Squitti and colleagues [10–12] showing elevated serum free copper in AD is not very controversial. It has been confirmed by others [50, 51]. Danzeisen et al. [36] criticize the method, but this criticism seems groundless. What is still to be debated and studied is what is the significance of this elevation? Does a higher serum free copper mean greater penetration of the blood-brain barrier and an elevation of extracellular toxic copper in the brain? Or alternatively, is it just a biomarker of other, critical, pathogenic pathways? It is our hypothesis that it is in the pathogenic sequence.

In conclusion, I believe our overarching hypothesis and subhypotheses, of a key role of inorganic copper from drinking water and supplements in causation of a new epidemic, an epidemic of AD, is well supported. I do not believe any of the contrary views, while some raise valid points to consider, have dealt fatal blow to my hypotheses.

I emphasize that at this point my concepts of copper, as one significant causative factor among others, are not finally proven. I believe they are important because if they are correct, they point to a method of at least partial prevention of AD, by lowering copper levels in drinking water and stopping ingestion of copper supplements. Those that take those steps now may benefit while waiting for final proof, which may take a long time.

Conflict of Interests

The author is currently senior vice president of Research and Development, Adeona Pharmaceuticals. Adeona is currently investigating zinc therapy for Alzheimer's disease, and part of the efficacy of zinc therapy might be in reducing copper levels.

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Research Article

Gender Effects on Plasma and Brain Copper

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The effect of gender on systemic and brain levels of copper is relatively understudied. We examined gender effects in mice and human subjects. We observed a trend to higher serum copper levels in female compared to male LaFerla "triple transgenic" (1399 ± 233 versus 804 ± 436 ng/mL, $P = 0.06$) mice, and significantly higher brain copper levels in female- versus male wild-type mice (5.2 ± 0.2 versus 4.18 ± 0.3 ng/mg wet wt, $P = 0.03$). Plasma copper was significantly correlated with brain copper in mice ($R2 = 0.218$; $P = 0.038$). Among human subjects with AD, both plasma copper (1284 ± 118 versus 853 ± 81 ng/mL, $P = 0.005$) and cerebrospinal fluid copper (12.8 ± 1 versus 10.4 ± 0.7 ng/mL, $P = 0.01$) were elevated in women compared to men. Among healthy control subjects, plasma copper (1008 ± 51 versus 836 ± 41 ng/mL; $P = 0.01$) was higher in women than in men, but there was no difference in cerebrospinal fluid copper. We conclude that gender differences in copper status may influence copper-mediated pathological events in the brain.

1. Introduction

Copper has been implicated in the pathological aggregation and neurotoxicity of beta amyloid (Aβ) [1] in Alzheimer's disease (AD). The evidence for "excess" circulating copper in AD has been reviewed recently [2] and copper-modulating therapies for AD are being evaluated [3, 4]. Although gender may influence the appearance of AD pathology, the effect of gender on copper status is relatively understudied. Some published reports have described gender differences in serum copper levels [5–7], but the effect of gender upon brain copper, which is more relevant to AD pathogenesis, has not been previously described.

We tested the hypothesis that gender modifies both circulating and brain copper levels, with potential consequences for AD pathology, using samples from an animal model of AD and from human subjects with and without AD.

2. Materials and Methods

2.1. Transgenic Mouse Studies. Breeding pairs of wild-type and "triple transgenic" (3xTg) mice [8] were generously provided by Dr. Frank LaFerla, and offspring were raised in the Portland VA Medical Center Veterinary Medical Unit. Mice were maintained on AIN93 diet from the time of weaning, with ad lib deionized water, so dietary intake of copper was closely regulated. For these experiments, 7 female wild type, 7 female transgenic, 4 male wild-type, and 3 male transgenic mice were used. At the age of 14 months, mice were euthanized with terminal collection of plasma and with rapid harvest of brain tissue. Copper levels were determined in brain (bilateral frontal cortex) and plasma by atomic absorption spectroscopy. All procedures were approved by the Portland VA Medical Center Institutional Animal Care and Use Committee.

2.2. Human Subject Studies. Individuals with AD as well as healthy control subjects were characterized by clinicians at the Oregon Health and Science University NIA-funded Alzheimer's center. AD was diagnosed according to NINDS-ADRDA criteria [9]. Healthy control subjects were tested with neuropsychologic battery and with interview of a collateral historian to ensure that they are genuinely healthy controls.

Table 1: Human subject characteristics.

	Young control	Middle-aged control	Old control	AD
n	11	16	15	38
Age in years (mean ± SEM)	30 ± 2	50 ± 1.8	74 ± 1.8	70 ± 1.2
Gender (% female)	19%	50%	47%	34%
MMSE	30 ± 1.3	30 ± 1.2	29 ± 1.2	17 ± 0.7

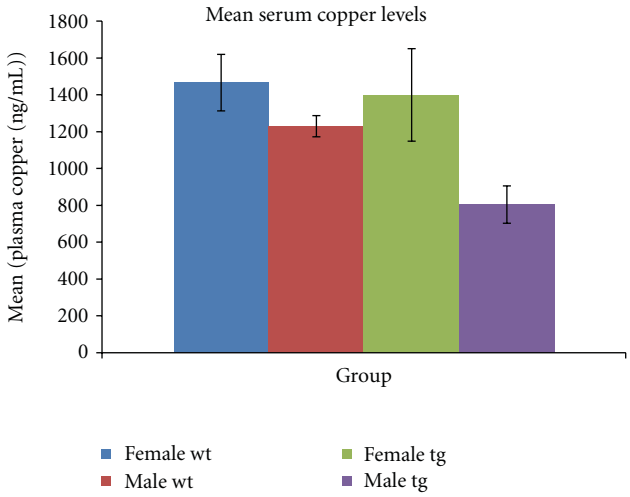

Figure 1: Serum copper levels (mean ± SEM) in triple transgenic (tg) and wild-type (wt) mice. Trends to higher serum copper in females are evident in both strains ($P = 0.06$ for female tg versus male tg; $P = 0.19$ for female wt versus male wt. $n = 7$ female wt, 4 male wt, 7 female tg, 3 male tg).

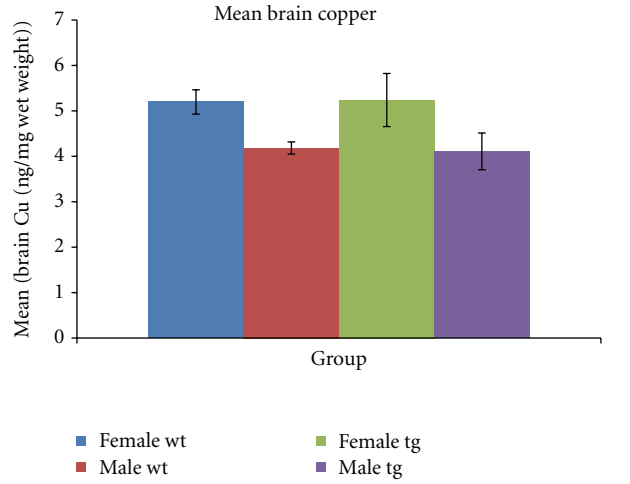

Figure 2: Brain copper levels (mean ± SEM) in triple transgenic (tg) and wild-type (wt) mice. Brain copper is significantly higher in female wt compared to male wt mice ($P = 0.03$), and a trend to higher brain copper in female tg compared to male tg mice is also evident ($P = 0.15$).

With appropriate Institutional Review Board approvals, subjects donated plasma by venipuncture and cerebrospinal fluid by lumber puncture. All lumbar punctures were performed in the AM fasting condition, in the lateral decubitus position. Both plasma and cerebrospinal fluid were frozen at −70 until copper measurements were performed by ICPMS.

However, plasma samples from AD patients and controls were collected in different tubes, with AD samples in heparin tubes and control samples in EDTA tubes (because the samples were initially collected under different protocols before being deposited in a repository). The use of EDTA (rather then heparin salt) collection tubes has been identified as a major source of variability in cross-sectional studies comparing blood levels across patient populations [2]. The analysis below consequently does not compare plasma levels across diagnostic groups.

2.3. Statistical Analysis. Group means were compared by two-tailed *t*-test or ANOVA, depending on the number of means being compared.

3. Results

(1) *Serum Copper in Mice.* Serum copper is increased ($P = 0.06$) in female (1399 ± 233 ng/mL) compared to male (804 ± 436 ng/mL) 3xTg mice, and a trend in the same direction is seen in wild-type mice (1467 ± 128 ng/mL in females and 1230 ± 169 ng/mL in males, $P = 0.19$) (see Figure 1).

(2) *Brain Copper in Mice.* Brain copper is significantly increased ($P = 0.03$) in female (5.2 ± 0.2 ng/mg wet wt) compared to male (4.18 ± 0.3, $P = 0.03$) wild-type mice, and a trend in the same direction is seen in 3xTg mice (5.2 ± 0.5 versus 4.2 ± 0.8, $P = 0.15$; see Figure 2). Plasma copper was significantly correlated with brain copper in mice (R2 = 0.218; $P = 0.038$).

(3) *Human Subject Characteristics.* Control subjects were characterized as "young" (age 20–40, $n = 11$), middle-aged (age 41–60, $n = 16$), and old (age ≥ 60, $n = 15$). The old controls did not differ from the AD subjects in mean age (see Table 1) or in percentage of women. Subjects with AD had mild deficits, illustrated by mean MMSE = 17 ± 0.7 (see Table 1).

(4) *Plasma Copper in Human Subjects.* Plasma copper was not correlated with age among control subjects or AD patients. Plasma and cerebrospinal fluid copper were not correlated in any group.

Plasma copper is increased in human female (1008 ± 51 ng/mL) compared to male (836 ± 41) control subjects ($P = 0.01$) when all age groups were combined. When the

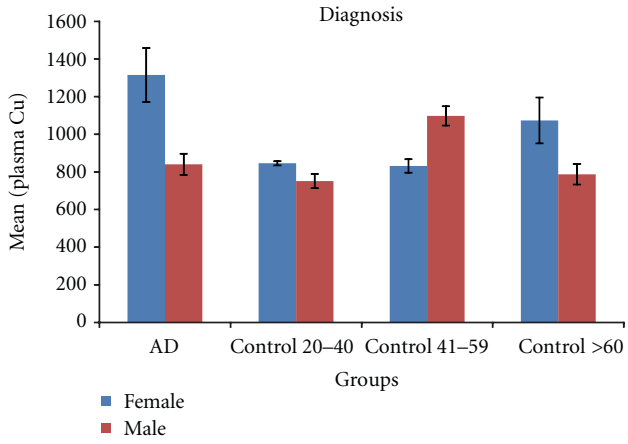

Figure 3: Plasma copper levels (mean ± SEM) in human AD and control subjects. Plasma copper is significantly increased in female AD ($n = 11$) compared to male AD ($n = 23$) ($P = 0.005$). There is a trend to increased plasma copper in old female control subjects ($n = 6$) compared to old male control subjects ($n = 8$) ($P = 0.09$) and in female middle-aged control subjects ($n = 8$) compared to middle-aged male control subjects ($n = 8$) ($P = 0.13$). When all healthy control subjects in each gender are combined, there is a significant difference in plasma copper ($P = 0.01$, see text).

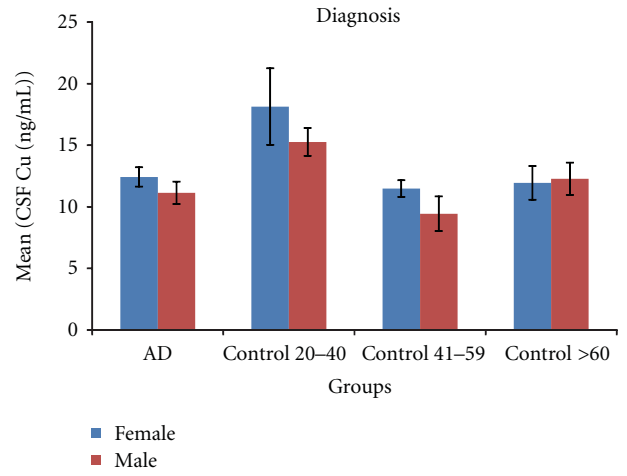

Figure 4: Cerebrospinal fluid (CSF) copper levels (mean ± SEM) in human AD and control subjects. CSF copper is significantly increased in female AD ($n = 16$) compared to male AD ($n = 25$) subjects ($P = 0.04$), but there are no significant differences in CSF copper between male and female healthy control subjects.

control subjects were divided by age group, trends to higher plasma copper in females were appreciated in the middle-aged and old control subjects, but not the young subjects (Figure 3).

Among subjects with AD, plasma copper was significantly increased in female compared to male AD subjects (1284 ± 118 versus 853 ± 81 ng/mL, $P = 0.005$).

(5) *Cerebrospinal Fluid Copper in Human Subjects.* CSF copper is increased in human female compared to male AD subjects (12.8 ± 1 versus 10.4 ± 0.7 ng/mL, $P = 0.01$). No gender-specific difference in CSF copper was seen in control subjects considered as a single group (12.2 ± 0.7 versus 11.6 ± 0.52; $P = 0.47$) or when considered by age group (see Figure 4). There was also no significant difference between AD patients and control groups in CSF copper, consistent with a recent meta-analysis on this topic [2].

4. Discussion

These findings in both mice and human subjects are consistent with a small number of publications in human populations which have found an effect of gender on serum copper, with higher copper levels in women than in men [5–7]. The possible confounding effects of dietary intake of copper [10] or the use of copper-containing supplements [2] has been emphasized in other studies of human subjects. However, the demonstration of gender effects in mice, with strict controls on copper intake which are not possible in human studies, strengthens the argument that these gender differences are due to gender-specific differences in copper trafficking rather than differences in dietary intake.

In some transgenic mice engineered to express AD pathology, female mice are more prone to AD pathology than male mice [11], and most studies examining the effect of gender on AD risk in human subjects [12–14] find an increased risk in women (although there is some controversy surrounding this point, with one study finding increased risk in women only after age 90 [15]). In light of these observations, it is interesting to speculate that gender differences in copper status, perhaps related in some way to iron-regulatory mechanisms related to menstruation, might modulate these apparent gender effects on AD pathology. Beyond speculation, gender effects on copper may also be important considerations in the design, conduct, and analysis of clinical trials of copper-modulating therapy for AD.

Further investigation of the consequences of gender-specific differences in copper status may be facilitated by clarification of the relationship between systemic and central nervous system copper. The significant positive correlation between serum and brain copper in the mice in this study supports the hypothesis that blood levels are relevant to brain levels of copper, and the concordance between serum and brain tissue results (with copper levels higher in females in both cases) provides further support for this view, at least with respect to frontal cortex.

However, since brain levels are not an option in living human subjects, these experiments used cerebrospinal fluid as a surrogate for brain tissue. In the case of subjects with AD, the effects of gender on plasma and cerebrospinal fluid copper were concordant, suggesting a blood-brain relationship similar to that seen in the mice. However, the absence of a correlation between human plasma and cerebrospinal fluid copper in both AD and control subjects suggests that either plasma copper does not reflect brain tissue or that cerebrospinal fluid is not an adequate surrogate for brain tissue in this instance. Alternatively, it may be

necessary to measure the proportion of CSF copper not explained by ceruloplasmin in order to appreciate plasma: CSF correlations, as reported by others [16].

5. Conclusions

These data add to existing evidence that female gender has an effect on blood levels of copper and provide new evidence that female gender may also have an effect on brain levels of copper. Gender effects on copper status may need to be considered in interpreting experiments, including clinical trials, which test the hypothesis that copper plays a role in AD pathogenesis and progression.

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