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Copper imbalance in Alzheimer's disease: Convergence of the chemistry and the clinic

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Abstract

In this perspective we list the many clinical, histopathological, genetic and chemical observations relating copper to Alzheimer's disease (AD). We summarize how the coordination chemistry of the APP/A β system is centrally involved in neuronal copper transport at the synapses, and that genetic variations in the gene coding for the copper transporter ATP7B cause a subset of AD, which we call CuAD. Importantly, the distinction between loss of function and gain of toxic function breaks down in CuAD, because copper dyshomeostasis features both aspects directly. We argue that CuAD can be described by a single control variable, a critical, location-dependent copper dissociation constant, K_d^c . Loss of functional copper from protein-bound pools reduces energy production and oxidative stress control and is characterized by a reduced pool of divalent Cu(II) with $K_d < K_d^c$. Gain of redox-toxic function is described by more copper with $K_d > K_d^c$. In the blood, the critical threshold is estimated to be $K_d^c \sim 10^{-12}$ M whereas at synapses it is argued to be $K_d^c \sim 10^{-9}$ M. The synaptic threshold is close to the values of K_d for Cu(II)-binding to A β , prion protein, APP, and α -synuclein, implied in copper buffering at the synapses during glutamatergic transmission. The empirical support for and biochemical and pathological consequences of CuAD are discussed in detail.

Keywords: Alzheimer's disease; copper; β -amyloid; K_d , ceruloplasmin; ATP7B

1. Introduction

Every day, thousands of people across the world are estimated to develop Alzheimer's Disease (AD)[1,2]. Despite its enormous prevalence and tragic consequences, currently available treatments only delay the disease by typically a few months[3,4], and the many recent failures of leading drug candidates have put renewed emphasis on alternative, more complete causal disease mechanisms[5–10]. The disease manifests first as mild cognitive impairment characterized by a gradual loss of episodic memory and leads to a slow, continuous impairment of cognitive abilities and life quality[3,11–13]. Neuron loss initiates in the cerebral cortex and commonly in the hippocampus[14].

Diagnosis of AD requires the observation of deposits of senile plaques outside the neurons and neurofibrillar tangles inside neurons[12,13]. The plaques contain oxidized and metal-bound β -amyloid ($A\beta$) peptides arranged in regular β -sheet fibril structures[15–17]. AD occurs most commonly with no apparent family history as "sporadic AD" (SAD); only a very small percentage relates to inherited mutations within a family, i.e. "familial AD" (FAD)[18]. FAD is caused by mutations in the genes coding for the β -amyloid precursor protein (APP)[19] and the presenilin isoforms PS1 and PS2; $A\beta$ is produced from APP by the action of β - and γ -secretases[20,21], and PS1/2 is the catalytic subunit of γ -secretase which produces $A\beta$ from APP together with β -secretase[22–26]. Thus, $A\beta$ is centrally implied both by biochemical hallmarks and genetic risk factors, leading to the dominance of the amyloid hypothesis in current drug development efforts[15,27,28].

Other biochemical hallmarks include molecular oxidative stress[29], impaired glucose utilization[30,31], and disturbed concentrations of metal ions, notably calcium[32–34], zinc[14,35,36], copper[37–41], and iron[42,43]. Each of these hallmarks have associated theories of pathogenesis assuming them to be causative, e.g. the tau[44–47], metal ion[48,49] and oxidative stress[50,51] hypotheses. Age is however the main risk factor of the disease[52] and should accordingly be central to any disease mechanism.

This review focuses on copper's increasingly recognized role in AD. Copper's importance to the central nervous system (CNS) is well-known and involves processes such as catecholamine synthesis, neurotransmission, adenosine triphosphate (ATP) synthesis, antioxidant defense, and myelin formation, which are briefly reviewed below and discussed elsewhere in detail[14,53–56]. Copper is crucial for CNS development and takes part in complex regulatory networks of proteins and pathways that maintain CNS homeostasis via the two copper-transporting P-type ATPases

(copper-ATPases)[57,58]. In recent years, it has become increasingly clear that impaired copper control plays a role in AD development and progression, mainly via an increase in serum Cu not bound to ceruloplasmin (noncp-Cu)[55]. Increases in noncp-Cu pertains to a percentage of AD patients, a copper-related AD subtype, which we call 'CuAD', characterized by high noncp-Cu levels and showing peculiar clinical characteristics linked to a perturbed copper homeostasis. These changes in noncp-Cu can be merged with the physical chemistry of copper-binding proteins which is also strongly related to AD[49,56,59,60]; the merging of these two fields to produce a united, consistent view of CuAD is the purpose of the present review paper.

2. The amyloid hypothesis and its main problems

The parallel between the plaque deposits in AD and the protein deposits in Creutzfeldt-Jakob disease inspired the hypothesis that the plaques in AD might be pathogenic[61]. When these plaques were found to consist primarily of aggregated A β peptides, the basis for the amyloid hypothesis was established[62] and further supported by the three major genetic risk genes of FAD coding for PS1/PS2 and APP[15,27,63].

The amyloid hypothesis is a "gain-of-function" hypothesis, i.e. it proposes that A β attains a toxic function in the brain that leads to disease[27,64]. Earlier it was commonly named the "amyloid cascade hypothesis", emphasizing a quantitative overload of toxic A β leading to AD[15,63]. The A β deposits in brains typically represent several years of total A β production[65]. Accordingly, drugs are pursued that prevent the formation of amyloids either by molecular interaction with A β [66–69] or by modulating its formation[70–76].

PS1 mutations often produce lower amounts of all A β isoforms than the wild type protein[20,77,78]. The 99-residue C-terminal fragment of APP (C99, produced by cleavage of APP by β -secretase (BACE1), is subsequently trimmed by γ -secretase in consecutive steps[79], resulting in A β peptides that vary in length, most notably A β ₄₀ and A β ₄₂[7,77], and the FAD mutations affect the precision of this cleavage[80,81] and tend to increase A β ₄₂/A β ₄₀ ratios[78,82–85], which, rather than any absolute amount of A β ₄₂ or A β ₄₀, is the main consistent feature of FAD mutations[28,78,86]. The long amyloids harbor additional hydrophobic amino acids and are thus more prone to aggregation[87,88] and more toxic to cells as these two features tend to correlate[15,89,90]. Thus, the current version of the amyloid hypothesis rests not on the total A β levels, but rather the local surplus of A β ₄₂ leading to disease. The importance of the A β ₄₂/A β ₄₀ ratio is supported by its correlation with clinical age of onset of PSEN1 mutation carriers[91].

Many (estimated 20–40%) cognitively normal older people possess enough senile plaques[92] to satisfy currently applied AD diagnostic criteria[93,94]. The measured signatures of neurodegeneration and cognitive decline do not correlate significantly with plaque load[4,64]. Neuron loss generally begins in specific parts of the brain (e.g. hippocampus) but APP and A β is broadly distributed, implying other disease modifiers[95,96]. Rather than the plaques themselves, the intracellular soluble oligomers of A β are particularly toxic and are considered the pathogenic culprits in the current version of the amyloid hypothesis[97–102].

The toxicity of A β oligomers depends substantially on their structure and size[103–105], and the specific pathogenic forms that allegedly causes AD remain unknown[90,106,107]. Also the mechanisms of action remain unknown although many have been suggested[108], including general seeding of protein misfolding[87,98,109], toxic redox reactions in their metal-bound forms[110–113], and A β forming membrane channels that facilitate metal ion transport[114–118].

The physiological structures of A β peptides are central for understanding their aggregation and natural functions[7,15,88,119]. The structural disorder of the peptide[120] makes measured toxicity very dependent on concentration, pH, ionic strength, co-solvents, and the time scale of the experiment performed[89,121]. Whereas aggregated fibrillar A β attains a regular β -sheet structure as seen in senile plaques, the free A β monomer has a disordered coil-dominated structure with only a small amount of helix and β -strand[120,122–124] as reflected in structural models with PDB codes 1BA4[125], 1IYT[122], 2LFM[126], 1Z0Q[123], and 1AML[127] (**Figure 1**). However, in non polar co-solvents such as hexafluoroisopropanol and close to membranes, the helix content of A β monomers increases[127]. When A β is in a membrane-like chemical environment, the C-terminal of A β forms an extended, potentially membrane-spanning helix, an observation that fits well with the widely documented membrane channel properties of A β [115,117,128–130]. The conformation, aggregation tendency, and potential function of A β monomers and oligomers depends on the coordination environment imposed by metal ions[131–133], as discussed further below.

Figure 1. A β structures derived from NMR experiments: **A)** A β_{40} in water (PDB: 2LFM)[126]. **B)** A β_{40} in a micelle membrane-mimicking environment (PDB: 1BA4)[125]. **C)** A β_{42} in an apolar environment (20% water, 80% hexafluoroisopropanol) (PDB: 1IYT)[122]. N-terminals are shown in blue, and C-terminals in red.

3. Brain copper homeostasis

Copper is an essential trace metal that is required for the activity of many enzymes and proteins; its homeostasis has been reviewed many times before but is briefly summarized below [14,134,135]. The redox cycling between its Cu(II) and Cu(I) oxidation states is utilized by numerous enzymes[14,136]. Copper is mostly transported and buffered in its Cu(I) state intracellularly, as for example bound to metallothioneins (MT), copper-transporter 1 (Ctr1), and ATPases ATP7A/B, but is functionally important as Cu(II)[137,138]. Vital functions involving copper directly in the catalytic active site include energy production via ATP synthesis in the mitochondria (cytochrome c oxidase, CCO), antioxidant defense (superoxide dismutase 1 and 3, SOD1 and SOD3), collagen maturation (lysyl oxidase), pigment synthesis (tyrosinase), and white and red blood cell production via hemoglobin synthesis and iron redox regulation (ceruloplasmin, haephestin)[14,137].

Figure 2. Established copper sites in some key proteins of the brain: (A) and (B) Copper-binding sites in the E2 domain of APP (PDB: 3UMK)[139]. (C) Copper (center, orange) in CCO (PDB: 5Z62)[140]. (D) Copper in the CuBD of APP (PDB: 2FK1)[141]. (E) Superoxide dismutase 1 (PDB: 2C9V)[142]. (F) Domain 2 of ATP7A (PDB: 1S6U)[143].

Examples of established copper sites in relevant proteins are shown in **Figure 2A-2F**. Most notably are the two copper sites in the extracellular E2 domain of APP (**Figure 2A and 2B**), one having a 4-histidine coordination sphere similar to that in SOD1 (**Figure 2E**), and the other E2 copper binding in a trigonal coordination geometry to one histidine, a carboxylate group of a glutamate residue, and a solvent water, with a potential coordinating methionine near-by[139]. Another copper site in APP is the so-called copper binding domain (CuBD)[144]; this copper binds two histidines and a tyrosine and two solvent molecules in a distorted trigonal bipyramidal geometry (**Figure 2D**)[141]. CCO, which is the terminal protein in the electron transport chain and thus required for energy production, contains both a Cu_A and Cu_B center. The Cu_B is coordinated by three histidines and is located uniquely close to a heme iron to assist in 4-electron-reduction of O_2 (**Figure 2C**)[140]. Finally, the copper site in the ATPase is characterized by

coordination to two cysteines in a distorted solvent-exposed coordination geometry; this structure is from solution NMR and thus less detailed (**Figure 2F**)[143].

In the brain, copper is also centrally involved in catecholamine synthesis via dopamine β -hydroxylase (DBH), catecholamine degradation via monoamine oxidase (MAO), neuropeptide synthesis via peptidylglycine α -amidating monooxygenase, glutamatergic synapse modulation, excitotoxicity, and myelin formation[14,135]. Via its antagonistic relationship to zinc managed by the metallothioneins, copper also affects zinc levels and associated processes[38,145–148], which again affects calcium levels[149,150]. Via ceruloplasmin, copper modulates iron uptake and distribution by influencing the oxidative state of iron, since ceruloplasmin catalyzes the oxidation of Fe(II) to Fe(III), which is then loaded into transferrin[54,151].

As discussed in detail below, the chemical and pathological features of disease tend to converge in the synapse. A simplified view of the copper homeostasis at the synapse is displayed in **Figure 3**. The healthy normal situation is shown in **Figure 3A**. The integral membrane protein Ctr1 imports Cu(I), which is then loaded onto copper-dependent enzymes via chaperone proteins such as copper-chaperone for SOD1 (CCS) and MTs. The two copper-transporting ATPases are encoded by the genes *ATP7A* and *ATP7B*. Both ATPase7A and ATPase7B remove excess Cu from cells, control influx of copper into vesicles, whereas primarily ATPase7B delivers Cu for incorporation into Cu-dependent enzymes. In the gastrointestinal tract, copper is pumped out by ATPase7A and then transported to the liver bound to albumin or α 2 macroglobulin or amino acids via the portal vein. In the liver, ATPase B incorporates copper into ceruloplasmin. The majority of the plasma copper is strongly bound to ceruloplasmin (called here cp-Cu), whereas the remainder is loosely bound and is exchanged among albumin, α -2-macroglobulin, amino oxidases, ferroxidase (II), SOD3, metallothioneins, and amino acids (called here noncp-Cu)[135,152,153].

In the central nervous system (CNS), astrocytes appear to play an important role in copper transport from the blood and cerebrospinal fluid (CSF) to the neurons[154,155], at the astrocyte foot (**Figure 3A**). At this site ATPase7A and ATPase7B control brain copper balance, starting from the early phases of development[156]. ATPase7A is expressed before birth, primarily in the hippocampus and cerebellum[157], then its levels decline. The protein is involved in synaptogenesis and axonal outgrowth and contributes to seizure resistance in hippocampal CA2 pyramidal cells[157]. ATPase7A is expressed in the basolateral membrane. While in the enterocyte it controls copper absorption into the body, in the endothelial cell of the blood brain barrier (BBB) it facilitates copper transport from the blood to the brain[158] and modulates synapses of

glutamatergic neurons[159]. Inactivation of ATPase7A in Menkes disease disturbs the transport of Cu into the brain, which leads to neurodegeneration[155,160]. ATPase7B is expressed at the apical side of endothelial cells of the blood cerebrospinal fluid barrier and facilitates copper transport into the CSF, as well as copper sequestration into the choroid plexus[156]. ATPase7B is expressed mainly in the hippocampus, in the cerebellum and brain cortex, and acts as a mediator of the synthesis of the Cu-dependent enzymes mentioned above[160].

Several other proteins locate at neuronal membranes and bind copper, most notably APP, α -synuclein (α -syn), and the prion protein (PrP), a glycoprotein located at synapse[161–163]. PrP is involved in copper uptake and efflux, and the metal facilitates PrPc internalization[164,165]. The interplay between copper and PrP appears to modulate glutamatergic transmission[166–169], and PrP also binds to A β [170]. Moreover, the binding of copper to the PrP increases its conversion to the infectious protease-resistant, β -sheet-rich form of the protein[171–173].

Figure 3. Simple overview of synaptic copper homeostasis. **(A)** Normal healthy brain. **(B)** CuAD brain. The main differences are shown as red copper and red crosses of probable impaired pathways. CCO = cytochrome c oxidase; SOD1 = Superoxide dismutase 1; MT = metallothionein; PrP = prion protein; CP = ceruloplasmin. In CuAD **(B)**, genetic (APP, PSEN1, or ATP7B) or life-style-induced changes in copper transport and balance leads to increased labile Cu, which disrupts ATP production (CCO), antioxidant defense (SOD1), and synaptic transmission.

Much of the bioinorganic chemistry of neuronal copper occurs at the synaptic cleft, where Cu(II) is released from the postsynaptic terminal upon stimulation of the N-methyl-D-aspartate receptor (NMDAR) during glutamatergic neurotransmission (**Figure 3A**). Cu(II) is presumably in a ‘free’ form when it is released at the synaptic cleft and modulates glutamate neurotransmission by down-regulating the NMDAR activity[156]. Copper is also released from presynaptic vesicles during transmission[174] in a recycling process regulated by PrP [159,167,168,175–178], which is a natural synaptic Cu(II) binding protein[164,166,171,172]. Due to the small volume of the synaptic cleft, Cu(II) can transiently reach micromolar concentrations and peak levels can be up to 100-fold higher[166].

While actual “free” exchangeable copper in the cytoplasm is virtually negligible at concentrations as low as 10^{-18} M [14,179,180], the distinction between loosely bound and strongly bound copper is important. The strongly bound pool of copper is characterized by a tight coordination geometry of non-solvent ligands as seen in active sites of copper proteins[137]. In the plasma this fraction is mainly bound to ceruloplasmin (cp-Cu). The remaining plasma Cu (noncp-Cu) is loosely bound to albumin, α -2-macroglobulin (transcuprein), and random peptides and amino acids and exchanged among them[136] and comes out from the liver; this type of copper crosses the BBB, is chelatable and engages in toxic Fenton-like reactions that produce very reactive radicals such as hydroxyl, which can contribute to oxidative stress in the aging neuron[14,179,181,182].

4. The critical K_d of CuAD

To differentiate the pathological and physiological Cu(II) pools, we use a threshold (or critical) dissociation constant K_d^c : Since $K_d = [Cu][P]/[CuP]$ where P is the copper protein, [Cu] is the “free” copper concentration and [CuP] is the complex, a small K_d corresponds to strong binding, whereas a large K_d represents copper that is relatively exchangeable. The Cu(I) oxidation state binds generally much more strongly to proteins, reaching attomolar K_d ($\sim 10^{-18}$ M or lower) in high-affinity copper storage and transport proteins such as MTs and Ctr1[183]. Since the copper concentrations and the affinities of the copper proteins vary with redox state and location, the relevant K_d^c is context-dependent, and we thus focus on the K_d^c of Cu(II) at the synapse which reflects the discussed pathology. Evidence suggests that dyshomeostasis will spread from location to location, as described below for the impact of *ATP7B* variants on brain copper homeostasis[184–

186]. The threshold K_d^c represents the affinity that separates, for the metal ion in the given location, the exchangeable solvent-accessible copper (all copper bound with $K_d > K_d^c$) from the strongly bound, inaccessible copper (all copper bound with $K_d < K_d^c$). K_d^c is a constant of the redox state and location, whereas concentrations of the two pools of copper can vary. We define the copper dyshomeostasis as the situation where the ratio $[Cu](K_d > K_d^c)/[Cu](K_d < K_d^c)$ increases.

As mentioned, the concentrations of copper vary by orders of magnitude dependent on redox state and location, and the amount of exchangeable copper varies according to the K_d of the local copper-proteins. In blood serum, the moderate-affinity copper-proteins albumin (10–15% of copper[187], mostly exchangeable, $K_d < 10^{-12}$ M[188]) and α -2-macroglobulin (5–15% of copper, which exchanges with albumin, $K_d \sim 10^{-12}$ M[189]) and the high-affinity ceruloplasmin (40–70% of copper, not exchangeable, $K_d = 10^{-15}$ M) ensure very little exchangeable Cu(II). The threshold of exchangeable Cu(II) in this location is then arguably close to K_d of albumin ($K_d^c \sim 10^{-12}$ M).

However, at the pathologically relevant synaptic cleft we argue that $K_d^c \sim 10^{-9}$ M: First, average $[Cu^{2+}]$ at the synapses (100–250 μ M)[190] is $\sim$ 100-1000-fold that of the CSF (0.3-0.5 μ M)[191] and the extracellular space ($\sim$ 1 μ M) [192] and the exchangeable $[Cu^{2+}]$ vary by 100-fold during synaptic transmission, whereas it is kept small and constant in blood and CSF. Second, K_d is surprisingly similar, typically 0.1–10 nM, for the disordered proteins suspected to be involved in synaptic Cu(II) transport and regulation, all being main culprits of neurodegenerative diseases, i.e. A β , PrP, and α -synuclein[193,194]. We argue that this is not a coincidence but reflects the K_d^c at the synapses. Third, the amount of copper in A β plaques ($\sim$ 440 μ M)[17] is similar to the average $[Cu^{2+}]$ at the synapses (100-250 μ M)[190] indicating that the K_d of A β is close to the threshold value for synaptic $[Cu^{2+}]$. Fourth, micromolar K_d values (10^{-6} M) in typical assays represent unspecific binding of labile metal ions such as Zn(II) with small binding free energy[14,193]. For Cu(II) at the same concentrations as Zn(II), a threshold K_d^c value 1000-fold smaller ($\sim 10^{-9}$ M) can be argued from K_d of complex formation to typical N-donor and O-donor ligands, i.e. the Irving-Williams series[195]. Fifth, we argue that the if ratio $[Cu](K_d > K_d^c)/[Cu](K_d < K_d^c)$ is increased in the blood as measured directly by noncp-Cu, this will also affect the ratio in other places. One example that we discuss further below is the analogy with failed copper incorporation into nascent ceruloplasmin upon defects of the *ATP7B* gene that cause Wilson's disease (WD), an autosomal recessive disease distinct from AD but with some biochemical features being similar to CuAD [54,160,196,197].

The K_d of the involved copper proteins may change by mutation or post-translational modification such as oxidations of sulfur ligands, and this will accordingly change the proposed pathogenic ratio $[Cu](K_d > K_d^c)/[Cu](K_d < K_d^c)$. Most notably, A β exists *in vivo* in various modified forms, with emphasis on N-terminal modified peptides of the type A β_{4-x} . Since these modifications affect the primary copper binding site, they also affect the K_d of copper and can reach much higher affinity than the full peptide and thus be functionally important[198–203].

The definitions above have centered on the thermodynamic binding equilibria of Cu(II). Cu(II) binding may be under kinetic control if release of copper becomes rate-limiting for equilibrating the copper pools described above. The lability is the appropriate term if the ligand-metal interaction is kinetically controlled, measured as the life time of the ligand's association with the metal ion or the rate constant of ligand substitution[204]. The thermodynamic (K_d) and kinetic (lifetime) definitions of pathological metal ions thus potentially differ, as indicated by some recent studies[205]. For high-affinity copper sites in proteins, kinetic control of copper pools may arise from barriers to release copper depending on the steric and electronic nature of the ligands. However, for the low-affinity synaptic proteins with $K_d \sim 10^{-10} \text{ M} - 10^{-9} \text{ M}$, exchange is very fast. NMR relaxation recently showed that the pathogenic A2V mutation of A β exchanges Cu(II) much faster than the protective A2T and wild type A β , despite having similar K_d values[206], suggesting a role of kinetics over thermodynamics in Cu(II) buffering. Rapid changes in $[Cu^{2+}]$ at the synapses can produce other barriers than induce kinetic control despite fast metal-ligand dissociation, for example due to slow vesicle copper release or membrane transport at the synapses. Under such conditions, low affinity proteins such as the synaptic A β , α -syn and PrP, will be much more adequate in restoring the copper pools, as copper is rapidly released from these sites on the timescale of synaptic transmission, supporting of a relatively high K_d^c at the synapses.

5. Clinical evidence: Copper dyshomeostasis in AD

Metal ion imbalances are a consistent feature of AD[15,33,34,37,50,104,182]. Several meta-analyses focusing on copper have been performed during the last 10 years merging data from more than 35 studies[207–211]; the most updated studies are shown in **Table 1**. The meta-analysis of copper in the brain shows that AD patients have lower levels of copper in their brains than healthy controls[185]. Meta-analyses monitoring the serum/plasma show increased copper in AD patients vs. controls[207,210–213]. This latter increase can be explained by the fact that the chelatable portion of copper (noncp-Cu) is increased in AD patients[209], in analogy with WD. A recent

study comparing directly WD and AD patients showed that while the two diseases differ in terms of serum copper and ceruloplasmin levels, they share increased levels of noncp-Cu, indicative of a failure a copper control[197]. Expansion of the weakly bound Cu(II) in AD coincides with decreased levels of functional protein-bound Cu(II) in the brain[208], as has been found in autopsy studies[214]. Analysis of copper in cerebral cortex Brodmann areas 22 and 46 showed reduced copper in AD patients, associated with 20% increase of labile copper in the same Brodmann areas[214], as also confirmed in other studies [214,215]. The results for copper differ from those for many other metal ions, including Zn, implying their possible antagonistic relationship[216].

While there is some disagreement on the ceruloplasmin concentration levels in AD[217], data on ceruloplasmin activity, which importantly reflect fully constituted copper-bound protein, are univocal, and largely similar for all the reported studies[151,191,217–220]. Ceruloplasmin is a 132 kDa multicopper oxidase that acts as an acute phase reactant scavenging oxygen-derived free radicals during inflammation states[54,187,221,222]. Ceruloplasmin is mainly synthesized in hepatocyte, where the ATPase 7B protein loads six copper atoms into apo-ceruloplasmin, generating an active holo-ceruloplasmin that is then transferred into blood circulation. It is also expressed in the kidney, mammary gland, placenta, and choroid plexus in the brain[187]. A ceruloplasmin isoform linked to the cell membrane by glycosphosphatidyl inositol (GPI) is expressed endogenously by the CNS and is abundant in astrocytes in the vicinity of neurons[223]. A key process catalyzed by ceruloplasmin is the oxidation of Fe^{2+} to Fe^{3+} , which reduces the availability of Fe^{2+} , promoting instead iron transport to various organs by transferrin, which accepts only Fe^{3+} [224]. Ceruloplasmin also scavenges H_2O_2 thus preventing the production of dangerous oxygen species[225]. Holo-ceruloplasmin synthesis strictly depends on the amount of available copper in the liver[224] and on ATPase7B function[135,187]. Consequently, reduced copper availability or ATPase7B protein dysfunction increases the fraction of apo-ceruloplasmin, which has no ferroxidase capability and is rapidly degraded in plasma[135,187].

The specific activity of ceruloplasmin decreases in AD and higher values in serum were shown to reduce risk of the disease[217]. The holo-form of ceruloplasmin in CSF[191], along with ceruloplasmin activity in serum[217,220] are decreased in AD patients. Low-molecular-weight fragments of ceruloplasmin derived from apo-ceruloplasmin degradation have been detected in AD samples and are suggestive of a defective activity of the ATPase7B copper pump[226]. Same mechanisms can be applied to the brain, positing a defective loading of the metal into other copper proteins and enzyme. Supporting this notion, AD risk variants of *ATP7B* (rs732774 and

rs1061472) have been found to cause abnormalities in the expression[227] and function of the ATPase7B[57]. As a whole this evidence suggests a disturbed activity of ATPase7B in loading the metal in copper proteins and enzymes in AD at liver but also at brain level.

Elevated levels of noncp-Cu in serum increase the risk of AD by approximately three-fold[228,229] and contribute to a worse prognosis of the patients[230]. Some AD patients display increased levels of noncp-Cu correlating with altered patterns of electrical brain activity and brain atrophy, along with pathological levels of A β and tau-protein in the CSF, and more severe clinical outcome[55]. In one study, subjects with mild cognitive impairment (MCI) with elevated noncp-Cu levels have a 3-fold increased probability of AD and faster conversion to AD[212]. Total copper and the ratio of copper to non-heme iron distinguished progressive MCI subjects from cognitively stable MCI subjects in a five-year follow-up study[37]. Other cross-sectional studies also demonstrated that noncp-Cu is higher in MCI than in healthy controls[219,231,232]. Large population studies suggest that copper imbalances occur at early stages of cognitive loss and AD development, and copper in the soil has been associated with increased mortality relating to AD (**Table 1**)[233].

Aside from AD, other studies have related copper levels to cognitive performance: A high copper diet combined with high levels of trans- and saturated fatty acids associated with a 3-fold increase in loss of cognition (**Table 1**, [234]). High copper plasma concentrations have been associated with loss of cognitive performance in normal women (**Table 1**)[235], and a poorer working memory and attention in children[236] and adolescents[237] (**Table 1**). A recent study found that copper in hair correlates with low cognitive abilities, high regional gray matter volume over widespread areas of the brain, and larger indices of low density in brain tissues (**Table 1**)[238]. As a whole, these studies indicate that elevated noncp-Cu is associated with loss of cognitive performance. These indications are further supported by a study investigating a neuropsychological battery of 19 tests in a cohort of healthy elderly women[239].

Several studies indicate that noncp-Cu may serve as a biomarker for specific sub-group of AD[240]. AD patients stratified on the basis of a noncp-Cu cut-off[219] have different clinical[241,242] and genetic features that relate to the frequency of specific variants of the *ATP7B* gene[243]. This evidence supports the existence of a detectable metabolic subtype of AD associated to copper abnormalities AD[240], called here CuAD. The AD risk variants of *ATP7B* (rs732774 and rs1061472) have been found to cause abnormalities in the expression[227] and function of ATPase 7B[57].

Many *in vitro* or *in vivo* studies have increased our knowledge about the likely causative mechanisms of copper dysregulation in AD[39,40,49]. *In vivo* studies in animal models demonstrated that small amounts (0.13 ppm) of Cu ingested through drinking water double plasma concentrations of noncp-Cu, reduce CSF A β clearance via the BBB in wild type mice, and increase A β production in a mouse model of AD[244].

It is known from many studies that cholesterol can enhance A β pathology in models of disease[245–248]. However, it was found that this tendency varied depending on the quality of water given to the animals, such that distilled water gave less pathology compared to tap water[249]. Strong evidence for a direct link between copper homeostasis and A β -related AD pathology came from the report that the reason is probably that trace amounts of Cu(II) (0.12 ppm) in water induce A β accumulation and senile plaque-like structures in the hippocampus and temporal lobe of cholesterol-fed rabbits, with associated retardation of learning ability[250]. These findings were later confirmed in another study with controls against other metal ions, Zn and Al, with no such effect[251]. These findings fit well with *in vitro* A β aggregation seen in many studies being caused by trace metals in contaminated laboratory glassware[252].

A further indication of Cu dyshomeostasis in AD comes from the fact that MT expression changes in AD[253][254]. These small cysteine-rich proteins naturally bind and buffer the concentrations of copper and zinc[145,179,255]. The MTI and MTII isoforms are found in almost all organs, whereas MTIII is predominantly expressed in the CNS. MTIII possesses a unique neuronal growth inhibitory activity distinct from the MTI and MTII isoforms[145,256]. MTI/MTII biosynthesis is induced by a range of factors including glucocorticoids, cytokines, reactive oxygen species, and metal ions. Expression levels of the three isoforms differ in AD: MTI and MTII expression is increased in AD[256], stimulated probably by the presence of free radicals, metal ions, and cytokines[256–258], whereas MTIII levels are decreased, notably in the temporal cortex[259]. MTIII shows extracellular growth inhibitory activity that antagonizes the ability of AD brain extract to stimulate survival and neuritic sprouting[260]. By a not fully understood mechanism, the extracellular Zn₇MTIII can protect neurons from toxic A β , through an interaction of free Cu(II) and Cu(II) bound to A β [257,261]. Accordingly, MTIII, but not MTI and MTII, have been reported to protect neuronal cells from Cu(II)-amyloid toxicity[257,258]. Similar mechanisms have been observed also for α -Syn and PrPSc proteins[258]: *in vitro* studies have demonstrated interaction of Zn₇-MTIII and Cu(II)-containing α -Syn and PrPSc indicating that Zn₇MTIII can be neuroprotective via copper transfer[257].

6. Biochemical evidence: APP/A β and copper transport

As expected from their major presence, APP and the APP-like proteins APLP1 and APLP2, serve natural roles in neurons. APP-knockout reduces synaptic proliferation[262]. Knockout of both APP and APLP2 simultaneously is lethal and indicates an important role of APP/APLP2[263,264] with a partial ability of mutual compensation[265]. The proteins are particularly important for synaptic function and affects synaptic plasticity[266–269] and are essential for hippocampus function and spatial learning of mice[270].

A β also seems to have normal functions in neurons[271–274]. Nanomolar concentrations tend to be neurotrophic, whereas micromolar concentrations as seen in cell assays are toxic[273,275]. The existence of a therapeutic window of A β may explain observations of both loss- and gain of function in AD, depending on study conditions[273,276,277]. Inhibition or depletion of A β ₄₀ by secretase inhibitors or antibodies is lethal to neurons[278]. A β depletion has also been shown to impair neuronal activity in mice[271]. More specifically, A β monomers seem to protect against copper- and iron-induced toxicity[272] and extracellular A β increases vesicle release probability in hippocampal synapses[274]. Together with the findings for APP, these findings point to an important role of APP/A β at the synapses.

Most of the proteins central to AD, including APP, A β , α -secretase, and the matrix metalloproteases that mainly degrade A β , have copper or zinc sites [14,279]. APP has multiple established metal binding sites[182,280–282] (**Figure 2A, 2B, and 2D**). The large extracellular part of APP contains the copper-binding domain (CuBD) [141,144,283]. A Zn(II) site is located within residues 181–200 of APP[284]. In addition, several other Cu(II) and Zn(II) sites are present in the E2 domain of APP-like proteins [139,285].

APP can reduce bound Cu(II) to Cu(I) [286], suggesting that APP may aid in transporting Cu(I) across the membrane similar to other proteins that enable Ctr1 Cu(I) transport, which requires a copper(II) reductase to bind Cu(I); in analogy to this function of APP, Ctr1 is cleaved from the membrane upon high concentration of copper to prevent uptake of copper[287]. Noteworthy, APP ablation in APP knock-out mouse model facilitates massive copper increases in the liver (80%) and in the brain (40%)[288]. This perturbation of copper efflux linked to APP ablation can be associated with an impairment of copper incorporation into copper proteins and the consequent increase of noncp-Cu that can reach the brain (up to 40%) [288] Previous data

indicate that 3% of serum noncp-Cu crosses the BBB in living AD patients[289], and consistent findings indicate a direct interaction of copper with A β in the CNS[244,250,290].

Further evidencing a function of APP in copper transport, APP knockout in cultured neurons leads to reduced sensitivity to copper toxicity [291]. In addition, overexpression of APP can both lead to increased Cu(II) reduction and uptake in cells[292]; however, overexpression can also cause intracellular copper deficiency in mouse brains[293], indicating that APP, either directly or by its cleavage product A β , also contributes to copper export. Other studies have shown that copper export caused by APP can be reduced by copper-chelators such as clioquinol[294]. The specific copper-binding domain of APP reduces toxicity from copper exposure[295], indicating a sensing-transport function to maintain intracellular copper concentrations at adequate levels. Inhibition of copper transporters increase A β and decrease expression of A β -degrading zinc proteases [296].

Interestingly, this copper buffering function is regulated by calcium levels: APP cleavage is regulated by increases in calcium concentration[297]. Secreted APP fragments can protect the neurons against excitotoxicity and reduce intracellular calcium [298]. In contrast to its plausible role in reducing Cu(II), C-terminal APP instead increases intracellular calcium levels which causes glutamate-based excitotoxicity[299]. Thus the buffering effect of APP/A β on copper and calcium seems to be antagonistic. Copper exposure to aged mice leads to A β accumulation and down-regulation of low-density lipoprotein receptor-related protein 1 (LRP1) [244], which is a transporter of A β [300] that also controls APP trafficking and processing[301,302]. However, dietary copper can also reduce A β production[303], and copper deficiency directly changes APP processing and A β secretion[304].

A β is a genuine metal-binding peptide with a typical N-terminal metal-binding sequence including three histidines (His-6, His-13, and His-14 using A β numbering[59,305]. Only two of them (His-6 and either His-13 or His-14) are thought to bind Cu(II) at any instance, and several ligand environments of the predominantly square-planar complex exists depending on pH[59,113,305–307]. At low pH (~6), only one histidine may bind Cu(II) probably due to protonation of the other[308]. Metal ions such as Cu(II) and Zn(II) mostly form 1:1 complexes with A β [282,309–311]. The K_d for copper depends on the conditions of measurement but is approximately 10^{-10} – 10^{-7} M[56,113,138,203,206,280,306,312–314], about 2-3 orders of magnitude more strongly binding than Zn(II), in accordance with the Irving-Williams series of metal ion binding[14,195].

Metal binding generally changes the aggregation tendency of A β , in some cases favoring fibrillar aggregates and in other cases oligomers[14,49,310,315–319]. The metal-rich fibrils resemble the senile plaques characteristic of AD[123,206]; however the Cu(II) bound states resemble early oligomers before aggregation, as estimated from thioflavin T assays[206,307]. The fibrils making up the senile plaques consist mainly of such extended sheets, packed with metal ions[17,123,320]. The coil-helix transition in low-dielectric environments (**Figure 1A-1C**) provides a structural rationale for its membrane channel function[116,128,321,322]. A β is known to form metal ion channels in membranes[116,117,128,130,323–325]. Such interaction involves conformational transition from disordered to helix, as favored upon Cu(II) binding[326] and facilitated by membrane-like micelle environments[122]. In combination, these various facts provide the structural-chemical basis for the suggested function of A β as a naturally disordered Cu(II) transport peptide[60], in agreement with the biochemical evidence of a copper-APP/A β relationship cited above. Metal binding most likely increases the ability of the peptide to associate with membranes, since the hydrophilic charged N-terminal becomes helical and less charged upon metal binding (from -3 to -1 when binding a divalent metal ion)[327].

As mentioned, A β is toxic and aggregation-prone at micromolar concentrations in typical assays[328,329]. However, this concentration corresponds to typically one year of total production and is 1000-fold higher than the normal sub-nanomolar concentrations (10^{-10} M) of A β [330,331]. Thus, the threshold concentration range separating beneficial and harmful A β is 10^{-7} – 10^{-9} M. Considering that A β typically forms 1:1 complexes with a K_d of Cu(II) of 10^{-10} – 10^{-7} M, 2-3 orders of magnitude more strongly than Zn(II), one can expect synaptic A β to buffer the Cu/Zn ratio in the synapses at 1/1000–1/100 approximately; this ratio is arguably important upon Zn and Cu release during glutamatergic neurotransmission.

β -secretase (BACE1) initiates production of A β by cleaving its N-terminal from APP[332]. The transmembrane region of BACE1 contains a highly conserved copper binding site[333–335]. Overexpression of BACE leads to reduced SOD1 activity due to loss of intracellular protein-bound copper[334,335]. Similarly, copper exposure leads to increased APP expression and A β formation by action of a copper sensing protein (CUTA) that interacts with the β -cleavage site of APP[336]. Recent work suggests that BACE1 functions via trimer formation upon cystine bridge formation to compartmentalize copper within the cell[337].

Further support for an in vivo relationship between APP/A β and copper comes from chelation studies. A variety of metal chelators, typically large complex phenolic N- and O-donor

systems such as clioquinol[338], coumarin[339] and benzylideneindanone[340] derivatives, and myricetin[341] reduce amyloid plaque formation[342], down-regulate APP expression and lower A β production with associated loss of intracellular loosely bound copper [343]. Strong metal chelators such as EDTA (estimated $K_d \sim 10^{-19}$ M from $\log K_f = 18.8$) reduce γ -secretase activity and thereby, production of A β [344]. Metal chelators reduce A β levels in cultured cells[345,346] and in *Drosophila* expressing human A β [347], and at the same time such chelators can improve the cognition of mice[348].

7. Molecular mechanisms of copper toxicity in CuAD

The mechanisms relating copper translocation in compartments prone to the disease are both loss of function (loss of bound copper in enzymes involved in anti-oxidant stress and energy production) and gain of function (induced protein aggregation, toxic redox activity of Cu-complexes), and both processes eventually contribute to neuronal death[60,277]. Even though the detailed mechanisms of these complex processes are still elusive, the many data reviewed in this work suggest that they act in concert in some AD patients.

Recent work shows that noncp-Cu has 95% specificity in detecting AD patients affected by CuAD[219], characterized by higher frequency of rs1061472 and rs732774 of the *ATP7B* gene, associated with susceptibility to AD due to copper exposure[243]. These gene variants are functional single nucleotide polymorphisms that modify expression and properties of ATPase7B[227]. ATPase7B loads copper onto nascent ceruloplasmin in the hepatocyte and onto the glycosphosphatidyl inositol (GPI)-ceruloplasmin expressed in the astrocyte, and slight defects in this process associated with *ATP7B* rs1061472 and rs732774 may explain the increased release of noncp-Cu in the blood[209] and decreases in ceruloplasmin expression and activity in both blood and CSF[135,187,191,217] and probably alterations in the expression of additional copper proteins of the brain[156].

Focusing first on the gain of toxic functions, when the pool of exchangeable copper increases in blood (as noncp-Cu) and in the brain, it may change the conformations of many intrinsically disordered proteins, as widely documented by Cu(II) binding leading to oligomer seeding and aggregation of A β [102,133,206,252,307,319,349,350]. Cu(II) may also engage in Fenton-like chemistry after reduction to Cu(I) in the reducing cell[14,351,352]. Overload of Cu(II)-A β molecules is also probable, particularly at the synapses where both are available at much higher than normal concentrations[53,159,274,353]. These complexes by themselves also have

both redox-toxic and potentially beneficial effects[181,199,206,314,354] that are so far not well understood in context, and separating the negative and positive effects of Cu(II)-A β (and other metal-A β complexes) is of major importance[60,277]. Efforts to modulate the aggregation tendency and redox toxicity of both amyloids alone and Cu(II)-A β complexes are accordingly of major current interest[74,75,355–359].

One underlying systemic variable of neurons that could potentially unify the risk factors of metal ion and protein dyshomeostasis is thus energy. The brain uses half of its energy for glutamatergic signaling and perhaps 30% of the remaining energy for protein synthesis and degradation. Cognition and memory are defined by energy requirements unique to the brain and are much more expensive in humans than rodents, ranging from 34% in rodents to 74% in humans[360,361]. The energy production decreases and the energy cost of maintenance grow monotonously with aging, providing a direct rationale for the age trigger of disease[362–364], with energetic abnormalities and energy efficiency (diabetes risk factors) being hallmarks of AD[365–367].

The catalytic reduction of molecular oxygen (O₂) to water (H₂O) by CCO generates the electrical gradient used by the mitochondria to create ATP, and mitochondrial dysfunction and associated quality control, which occurs upon impaired energy production, is a hallmark of AD and other neurodegenerative diseases[363,368–370]. If copper is lost from the bound functional pools, mainly from CCO, this energy production will be impaired, such that energy surplus for cognition may ultimately be affected. At the same time, noncp-Cu can increase stress of misfolded proteins that in turn can increase the cost of turnover[371,372]. A β 's role in this vicious circle may possibly be to restrain and buffer the increased levels of oxidative stress and the disruption of the ionic gradients due to copper dysfunction (**Figure 3B**).

8. Analogies to other Cu-related diseases

WD is the paradigmatic disease of noncp-Cu overload in tissues and organs caused by failure of copper incorporation into ceruloplasmin in the hepatocyte[196,240]. Upon *ATP7B* mutations noncp-Cu increases in the liver, brain, and the cornea of the eye (Kayser-Fleischer rings), producing oxidative stress and tissue damage[135,373]. As a consequence WD has a broad spectrum of symptom presentation. About one third of cases present with liver disease, another third with neurological manifestations, and the last third involves psychiatric and behavioral abnormalities[196,374–380]. In WD brains, the labile component of Cu is increased and deposition

occurs particularly in the basal ganglia, associated with gliosis, neuronal loss[381] and features that resemble Parkinson's disease[382].

Mass screening in Korea suggests that WD may be much more common than previously estimated and reach 1:3000 to 1:1500 cases[377,383]. In line with this, a recent study reported an unexpected rate of about 1 in 40, higher than the 1:90 previously reported, of *ATP7B* heterozygote mutation carriers in UK, yielding a prevalence of 1 per 7000 inhabitants[377,384]. The large discrepancy between the estimated genetic prevalence of WD based on *ATP7B* mutation frequency and the number of clinically diagnosed WD patients, which is significantly lower, motivates the hypothesis that *ATP7B* mutations may increase the likelihood of other neurological or mental disorders, e.g. AD. In line with this hypothesis, some *ATP7B* mutations have been identified in AD patients[385]. Multiple *ATP7B* variants modify copper homeostasis and the risk of the disease, and at least in a percentage of patients, specific loci of the *ATP7B* (primarily K952 and R832) contribute to the global susceptibility of developing AD (CuAD) [386]. *ATP7B* Genetics appears to drive a large part of the association between copper abnormalities and brain development and function. The BREATHE project highlights an interaction between specific *ATP7B* variants (including *ATP7B* R832) and alterations of basal ganglia structure and its function in the brain [387] (**Table 1**). Multiple *ATP7B* gene variants apparently interplay with diet or life style to modify susceptibility and disease phenotype, and could thus provide correlations to nutrition during aging, when the copper requirements are reduced relative to other phases of life[388].

9. Combining the facts into a simple model of AD due to copper dyshomeostasis

The many studies of the role of copper at the synapse suggest that APP can play a role in reducing Cu(II) to Cu(I) to enable Ctr1 function, and that ATP7A/B can facilitate copper translocation into vesicles, which can then be released at the synaptic cleft as part of the glutamatergic transmission that occurs also in the hippocampus at the pyramidal CA1 synapse[159,167,174] (**Figure 3A**). In this model PrP and APP can serve as buffer proteins that control [Cu(II)] within the synaptic space, where it can reach micromolar concentrations and is subject to major variations[166,389]. The threshold binding strength of these Cu(II) ions at the synapse is of the order of 10^{-9} M, which is, incidentally, also the typical neuronal concentration of A β , which also tends to localize at synapses[353,390]. A β binds copper preferably with 1:1 stoichiometry[199,203,305,391] and interacts with the membranes to form calcium channels[115,116,128,324].

The evidence summarized above strongly suggest that copper dyshomeostasis can interfere with this basic synaptic process in at least a subset of AD patients. We define this dyshomeostasis as a change from strongly bound synaptic copper pools ($K_d < 10^{-9}$ M) to loosely bound pools ($K_d > 10^{-9}$ M) over time, with a correlated excess noncp-Cu in the blood. The resulting change in the proposed pathogenic ratio $[Cu](K_d > K_d^c)/[Cu](K_d < K_d^c)$ would make it more difficult for the copper buffer proteins to restore copper pools. A concomitant loss of functional bound copper in copper proteins will impair SOD1 activity and ATP synthesis by CCO, causing energy depletion and oxidative stress (**Figure 3B**). The disruption of copper homeostasis at the synapse can affect the synaptic strength: Application of physiologically relevant concentrations of 1 μ M $CuCl_2$ in rat hippocampal slices inhibited long-term potentiation, which is one of the basic processes of learning and memory[392]. Similar pathology is observed in rats fed with a high copper diet[393] or injected with copper[394].

A previous model of copper involvement in AD[240] held that the neurovascular unit has a primary role in regulating the transport of copper from the blood to the interstitial space in the brain. The concentration of Cu in the interstitial space is primarily controlled by the astrocytes and the other cells of the neurovascular unit[155]. ATPase7B and ATPase7A and Ctr1 have primary control over Cu levels (**Figure 3A**). Specific variants in the *ATP7B* gene (e.g. rs1061472 and rs732774, but additional variants may exist) affect the expression and function of the ATPase7B pump[57,136,227] (**Figure 3B**). Overtime, *ATP7B* dysfunction may increase noncp-Cu in serum plasma and reduce the copper loaded into copper proteins (including transporters) and enzymes, as exemplified by reduced ceruloplasmin specific activity[135,187,191,217]; in the brain these processes can correspond to copper depletion and probably a general loss of function of copper proteins including ATPase7B, CCO, SOD1, DBH, and MAO[156]. In particular, DBH catalyzes the formation of noradrenaline from dopamine. Midbrain catecholamine diffuse neurotransmitter systems have broad effects that affect the entire cerebral cortex. Apart from the disturbed equilibrium of the neurotransmitters (dopamine, noradrenaline, serotonin) which almost certainly would affect brain function, increased levels of dopamine may contribute to oxidative stress via autoxidation and MAO activity, generating H_2O_2 that can directly react with noncp-Cu in Fenton-type reactions producing the extremely reactive hydroxyl radical ($OH\bullet$)[14,395].

The many data indicating that the natural APP/A β function relates to copper (and possibly zinc) transport at the synapses put the amyloid hypothesis in close and logic relationship to the model described above. Since the APP/A β system aids copper transport at the synapses, the

disruption of A β levels by PSEN or APP mutations would contribute to a disturbance of the copper homeostasis, as indicated in **Figure 3B**[60,277,396]. In this model, APP and PSEN mutations, the main genetic risk factors of familial early-onset AD, cause disease by a partial loss of function that manifests at high age when the overall copper homeostasis is already stressed. We also note that mutations in SOD1 cause Amyotrophic lateral sclerosis[397–399], a severe motor neuron disease which we speculate, because of its many clinical similarities to AD, share some of the pathways shown in **Figure 3B**, notably those relating to copper-constituted functional SOD1.

10. Conclusions

From the evidence collected it is clear that copper dysfunction pertains to a percentage of AD patients, a condition we call here CuAD. This copper dysfunction can explain a fraction of the global susceptibility for AD. In addition to the pathological and clinical evidence, there is also substantial biochemical support for a strong link between copper status and APP/A β , with current evidence pointing toward a function in copper transport across neuronal membranes, either directly or indirectly, as a support of Ctr1. Thus the main hallmark of AD, A β , is a proficient copper binding peptide with a very broad range of data now documenting how copper binds and modifies A β structure, aggregation, and toxicity.

For the subset of AD associated to copper dysfunction, causes relate to four processes that can be summarized as follows: i) increased frequency of specific *ATP7B* variants, ii) increased levels of noncp-Cu, and iii) decreased specific activity of ceruloplasmin; iv) decreased protein-bound copper into the brain, such as ceruloplasmin, ATPase7B, MT, CCO, SOD1, and DBH[400–403]. These processes can influence each other but standing alone they can still increase the risk of the disease. They can trigger a cascade of processes that involve neurotransmitter alteration, oxidative stress, and disruption of energy production, eventually leading to cell death. Large population studies and meta-analyses support the proposed pathways shown in **Figure 3**.

We suggest that the transition from bound functional copper to “free” chelatable noncp-Cu is defined by a location-dependent threshold binding constant for pathogenicity which is approximately $K_d^c \sim 10^{-12}$ M in blood and $K_d^c \sim 10^{-9}$ M in synapses, where stronger binding represents innocent functional copper and weaker binding represents, at least to some extent, the redox-active gain-of toxic function. A large reduction potential of the Cu(II) concomitant with access to reductants such as ascorbate produces the Cu(I) state that may undergo Fenton-like

reactions[404]; it is quite interesting that Cu(II)-A β , which is also a weakly bound copper-system, can do the same[202,405]. At oxidative conditions hydroxyl radicals are not formed to a great extent[352] but in the reducing environment of neurons they are likely to do so, as well as reacting with sulfur groups of proteins[404]. This threshold represents the shift from specific (controlled and contained) to nonspecific binding in physical chemical binding assays[14,193]. The K_d of A β for copper is 10^{-9} M which is strong enough to bind and sequester the “free” copper but weak enough to release it after transport. Copper binding to A β changes its conformation and increases its hydrophobicity, probably thus enabling interaction with membranes. Mutations in PSEN1 and PSEN2 that cause FAD change the production of A β peptides which may affect the specific transport capacity of copper-A β across the neuronal membrane[60].

Finally, we would like to emphasize that Professor Brewer, based on epidemiological and demographic data that are completely orthogonal to the chemical, genetic and clinical data presented here, has put forward a theory that a subset of sporadic AD can be caused by increased copper exposure. He reaches this suggestion based on data from developed and industrialized countries with and without copper piping in a historical context and from toxicological studies of the A β pathology induced by normal copper exposure in drinking water[406–408]. Given that the biochemical mechanisms would be similar to those described here and thus strongly documented, we suggest that this possible contribution to sporadic AD is explored further.

Conflicts of interest

KPK declares that he has no commercial or noncommercial conflicts of interest relating to this work. RS is Chief Scientific Officer of IGEA Pharma N.V. and has some shares in Canox4drug and IGEA Pharma N.V., but does not receive monetary compensation.

Table 1. List of the most updated meta-analyses of copper in Alzheimer's disease, in developmental disorders, and epidemiological studies of copper in cognition.^a

Most updated meta-analyses copper in Alzheimer’s disease							
Reference (Author, year)	Cu biomarker	Tissues sample sources	Studies analyzed	Number of AD Cases	Number of controls	Trend	p value
Li et al. 2017[211]	Cu	serum	35	2128	2889	increase	<0.0001
Squitti et al. 2014[209]	nCp-Cu	serum/plasma	10	599	867	increase	<0.001
Bucossi et al. 2011[207]	Cu	serum/CSF	5	116	129	no diff in CSF, increase in serum	
Schrag et al. 2011[208]	Cu	brain	7	115	123	decrease	=0.0003
Meta-analyses of copper in neurodevelopmental disorders							
Reference (Author, year)	Condition	Tissues sample sources	Studies analyzed	Number of Cases analyzed	Number of controls	Results	
Saghazadeh et al. 2018 [409]	febrile seizures	Serum/plasma	4	99	92	Cu increases in patients	
Saghazadeh et al. 2015 [409]	epilepsy	Serum/plasma	10	934	899	No changes in Cu	
Epidemiological studies of copper and cognition							
Reference (Author, year)	Type of analysis	Number of Cases analyzed	Results				p value
Morris et al. 2006 [234]	Longitudinal, 9 years follow- up	3718 (CHAP study)	Among persons whose diets were high in saturated and trans fats, higher copper intake was associated with a faster rate of cognitive decline (participants in the highest fifth of cu intake combined with a diet high in saturated fats lost cognition three times faster than expected).				<0.01

Lam et al. 2008 [235]	Cross-sectional, and cores on 11 of 12 cognitive function tests Plasma analysis	1551 (Rancho Bernardo Study)	In women copper concentrations had inverse linear associations with Buschke total, long and short-term recall and Blessed scores (cognitive test).	<0.001
Shen et al. 2014 [233]	Copper concentration in soil in relation to annual mortality of AD	Chinese general population, from 1991-2000, in 26 provinces, 3 districts	relative risk of mortality in the regions with the highest copper concentrations (60-80 mg/kg) was high [OR: 2.63 (95% CI: 2.626–2.642)]	<0.001
Zhou et al. 2016 [410]		826 children	significant association between high copper levels and poorer working memory in boys (unstandardized regression coefficient (b) = -0.099, robust standard error (s.e.) = 0.009, t = -10.99, p = 0.008, 95% CI (-0.138, -0.060).	
Kicinski et al. 2015 [237]			A two-fold increase in blood Cu was associated with a 0.37 standard deviation decrease in sustained attention (95% CI: -0.67 to -0.07, p=0.02) and 0.39 standard deviation decrease in short-term memory (95% CI: -0.70 to -0.07, p=0.02).	
Pujol et al. 2016 [387]		2836 children aged from 8 to 12 years.	The results establish an association between environmental copper exposure in children and alterations of basal ganglia structure and function.	P<0.001
Takeuchi et al. 2019 [238]	gray matter volume (rGMV), mean diffusivity (MD), and cognitive performance	924 healthy young adults	Cu in hair associates with aberrant cognitive functions, greater rGMV in extensive areas, greater MD (which are indicative of low density in brain tissues) in subcortical structures in the healthy young adults	P<0.0001 for rGMVs

Alemaný et al. 2017[411]		1,645 scholar children from the BREATHE project	<i>ATP7B</i> rs1061472 (P for interaction 0.016) and <i>ATP7B</i> - rs1801243 (P for interaction 0.003) polymorphisms modified the association between indoor copper exposure and inattentiveness.	
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^a Risk is reported as OR=Odds ratio; CI=Confidence Intervals.

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