6

The Role of Trace Metals in Alzheimer's Disease

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Abstract: The extracellular aggregation of insoluble protein deposits of amyloid-β (Aβ) into plaques and the hyperphosphorylation of the intracellular protein tau leading to neurofibrillary tangles are the main pathological hallmarks of Alzheimer's disease (AD). Both Aβ and tau are metal-binding proteins. Essential trace metals such as zinc, copper, and iron play important roles in healthy brain function but altered homeostasis and distribution have been linked to neurodegenerative diseases and aging. In addition, the presence of non-essential trace metals such as aluminum has been associated with AD. Trace metals and abnormal metal metabolism can influence protein aggregation, synaptic signaling pathways, mitochondrial function, oxidative stress levels, and inflammation, ultimately resulting in synapse dysfunction and neuronal loss in the AD brain. Herein we provide an overview of metals and metal-binding proteins and their pathophysiological role in AD.

Keywords: Amyloid beta; copper; iron; metal-binding; zinc

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INTRODUCTION

Essential trace metals and those with biological functions (biometals) play a vital role in many physiological processes in the human body. As free ion, some of them can participate in cellular signaling pathways, while bound to proteins they may have a structural or regulatory role in protein folding and function. The fact that about 10% of the genes in the human genome encode for proteins with zinc (Zn)-binding motif points to the evident dependency of biological processes on this trace metal. This number of Zn-binding proteins is not even accounting for Zn coordinated between two proteins in protein–protein interactions (1). In addition to Zn, several proteins and processes depend on other essential trace metals, the most important of which are iron (Fe), manganese (Mn), copper (Cu), and selenium (Se) (a metalloid). The average human body contains about 4.2 g Fe, 2.3 g Zn, 0.072 g Cu, 0.015 g Se, and 0.012 g Mn (2). However, the distribution of trace metals can vary depending on the organ considered. In the human brain, Fe is the most prevalent trace metal, which can be found both as heme (bound to hemoglobin in blood) and non-heme Fe. Heme-bound Fe may be a major contributor to the overall concentration. Therefore, Zn, the second most prevalent metal, may play an even more prominent role in the brain, which is underlined by its function as neurotransmitter/neuromodulator (3). Additionally, within the brain, some trace metals are enriched in particular brain regions (Figure 1). For example, the hippocampus is a brain region that is high in Zn, while the nucleus caudatus has higher levels of Fe than several other brain regions (4, 5). This unequal concentration of trace elements in different tissues demands a tightly regulated distribution. Given that charged molecules such as metal ions cannot freely pass the cellular membrane, a plethora of transport proteins evolved, with very specific regional and also developmentally and environmentally dependent expression. Especially, the regulation of metal concentrations in the brain faces a tight control at the level of the blood–brain barrier (BBB), a barrier composed of endothelial cells of the brain capillaries, pericytes, astrocytes, and the basement membrane (6). Together, they form a functional unit, mediating the exchange of trace metals between neurons, capillaries, and glia, while protecting against neurotoxicity of non-essential trace metals or excessive levels of essential trace elements. A specific set of transporters allows the crossing of trace metals into the brain. For example, only for Zn, 24 different transport proteins are known in humans (7), which allow the establishment of zinc homeostasis in tissues.

The maintenance of a balance between biometals is complicated by the influence different metals have on each other. Their concentration is regulated by complex interactions between trace metal ions and their ligands. For example, due to their physicochemical nature, Zn and Cu are known to compete for the binding sites of some transporters and metal-binding proteins, resulting in an antagonistic relationship, where low levels of Zn increase Cu levels and vice versa (8). Due to these interactions, the loss of, or increase in, one trace metal can lead to the establishment of a completely new biometal profile affecting many other trace metals of a system (9).

Metal homeostasis can be challenged in many ways. In fact, our body is not only exposed to essential trace metals. Through the environment (e.g., air, food,

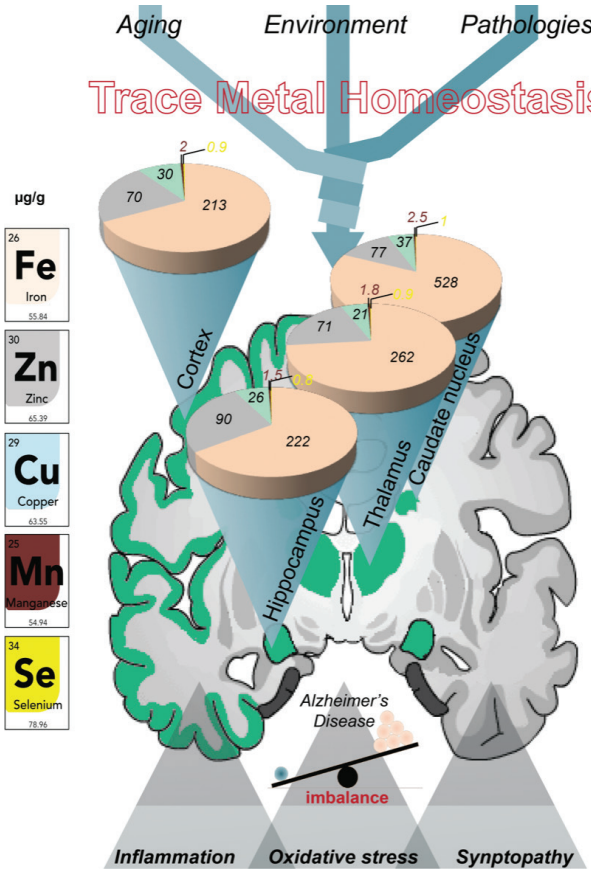

Figure 1 Trace metal concentration in different brain regions. Within the brain, trace metals are unequally concentrated in different brain regions. The figure shows the concentrations in µg/g brain tissue in the human cortex, hippocampus, thalamus, and caudate nucleus for Fe, Zn, Cu, Mn, and Se. This metal homeostasis can be challenged by environmental factors, the presence of pathologies such as aggregates of metal-binding proteins, and aging.

medical devices, and cosmetic products), other nonessential trace elements such as lead (Pb), mercury (Hg), and aluminum (Al) may enter our system. Indeed, these metals are present in all humans at low levels (e.g., 0.060 g Al, 0.012 g Pb, and 0.006 g Hg). Some of these metals are currently reported to have no or little effect on the body [e.g., titanium (Ti)], while others can produce adverse effects even at concentrations slightly above the normal background levels [e.g., Pb, Hg, and cadmium (Cd)]. These toxic effects are usually due to a chemical nature similar to that of an essential metal that allows binding to metal-binding sites of metal transporters and other proteins, leading to competition with essential trace metals. However, toxic metals are often not able to produce the biological effect of essential metals, and therefore, they act antagonistically. Recent evidences put synaptic signaling, synapse formation and

plasticity, oxidative stress, inflammation, and protein aggregation at the forefront of disease-relevant processes caused by abnormal trace metal homeostasis (Figure 1).

The enrichment of biometals in a tissue may occur through several mechanisms, such as mutations in metal import and export proteins, proteins buffering metals through transient binding (e.g. metallothioneins) (10), and also the abnormal accumulation of metal-binding proteins that occurs in several neurodegenerative diseases such as Parkinson's disease (alpha-synuclein protein) (11) and Alzheimer's disease (AD).

In AD, a contribution of abnormal trace metal homeostasis and signaling has been extensively reported (9). However, changes in trace metals' levels in AD are complex and can rarely be directly associated with systemic alterations that can be measured in easily accessible biosamples such as serum. The most likely reason for this is the ability of senile plaques to sequester specific metal ions that in turn become mislocalized instead of decreasing or increasing systemically. Cu, Zn, and, to a lesser extent, Fe are known to associate with senile plaques made of beta-amyloid (Aβ) protein (see below). It is hypothesized that this association causes several biological effects. For example, sequestration of Cu and Zn into plaques leads to an abnormal distribution of these metals, initially resulting in a deficiency of Cu and Zn in the vicinity of plaques (12, 13) and not throughout the whole brain.

Therefore, findings concerning alterations in metal ions in AD are highly dependent on the tissue and resolution used for analysis. Regarding essential metals, although results vary in some studies, Mn, Cu, Fe, and Zn seem to show an inverse correlation with senile plaque load and thus a decrease in the cerebrospinal fluid (CSF) (14) of AD patients.

The accumulation of trace elements, including Al, Pb, Hg, Cu, and Fe, has been implicated in AD through an increase in oxidative stress (15). In particular, a disruption in the homeostasis of Cu and Fe, two redox-active metals, may increase lipid peroxidation, and the oxidative damage to neurofibrillary tangles (NFTs), senile plaques, and nucleic acids (16). Oxidative stress is induced by an imbalance in the redox state, involving the generation of excessive reactive oxygen species (ROS) or the dysfunction of the antioxidant system (17). Cu is a potent mediator of the highly reactive hydroxyl radical (OH•) and is highly concentrated in senile plaques. Consequently, Cu contributes to the increase of oxidative stress in AD. In addition, increased levels of Fe, transferrin, and ferritin may contribute to NFT formation, possibly due to the binding of Fe to the tau protein. In the brain, oxidative stress may cause serious damage via several mechanisms, including the release of excitatory amino acids and neurotoxicity (18). Although Zn is redox-inert, Zn signaling plays a role in the regulation of proteins (e.g., enzymes kinases and phosphatases) controlling redox-signaling pathways. Therefore, while not acting as an electron donor, Zn plays a role in redox biology, where zinc, in general, is considered as an antioxidant. However, these indirect antioxidant-like effects are present only in certain conditions and both a lack and excess of Zn can result in pro-oxidant effects (19).

In addition to changes in trace metal homeostasis resulting from AD, it is likely that alterations may also facilitate and trigger the development of AD pathology. For example, it has been shown that the levels of Zn decrease during

aging as a result of more restricted food choice (20, 21), which may facilitate inflammatory processes (22), increase oxidative stress (19), and decrease memory as seen in several animal models for Zn deficiency (23). More importantly, key proteins involved in the etiology of AD, and especially early-onset AD (familial AD), such as amyloid precursor protein (APP), presenilin 1 (PS1), and presenilin 2 (PS2), have been shown to bind to or regulate metals. For example, PS are important for cellular Cu and Zn turnover (24). Further, metals have been shown to interact with the two major disease-related proteins of AD, namely Aβ and tau.

METAL INTERACTIONS WITH APP

The APP is expressed in various tissues of the human body, in particular in the brain. Its general function within the brain has been linked to neurite outgrowth and neuronal cell migration (25). However, it becomes increasingly evident that APP can be considered to act as a metalloprotein, which is involved in the regulation of Cu, Fe, and ferroxidase homeostasis (26). Recent studies indicate furthermore that metals are involved in the proteolytic processing of APP.

APP displays two putative metal-binding sides, which are located within the E1 (124–189, APP770 numbering) and E2 (376–554) domains (27, 28). Cu binds to APP between residues 142 and 166 (29). It has been demonstrated that the two Cu-binding residues 149 and 151 are involved in the metabolism, folding and stability, and homodimerization of APP (30). Besides, Cu ions have been shown to promote cell surface localization of APP (31). Furthermore, it has been shown that cellular Cu levels can influence the expression of APP in vitro at both gene and protein levels (32).

APP also displays an evolutionary conserved Zn-binding site between amino acid positions 170 and 188 (33, 34). The binding of Zn to APP has been reported to play a similar role as Cu-binding in the homodimerization of APP (35) (Figure 2A).

Fe is involved in the direct regulation of APP translation. The APP mRNA displays an Fe response element (IRE) in its 5'-untranslated region (5'-UTR) sequence (36), and APP levels increase after a rise in cytosolic free Fe levels (37). Additionally, APP has been suggested to be involved in Fe export in the brain through the stabilization of ferroportin (Fpn). Deletion of APP in vitro in primary neurons impairs Fe export, which can be fully restored by the addition of APP (38).

Aβ is derived from APP by the sequential proteolysis by β- and γ-secretases. Metals have also been shown to indirectly influence Aβ generation by modifying the proteolytic processing of APP (28). Interestingly, all three secretases (α, β, and γ) involved in the enzymatic cleavage of APP interact with metal ions. The enzymatic activity of the α-secretase TACE is regulated by a "cysteine switch" motif, which is based on an intramolecular bond between cysteine (Cys) and a Zn atom in its catalytic site (39). Furthermore, the major β-secretase involved in APP processing displays a Cu-binding site in its C-terminal domain (40). Further, Zn has been shown to enhance the synthesis of PS1, the active subunit of the γ-secretase (41).

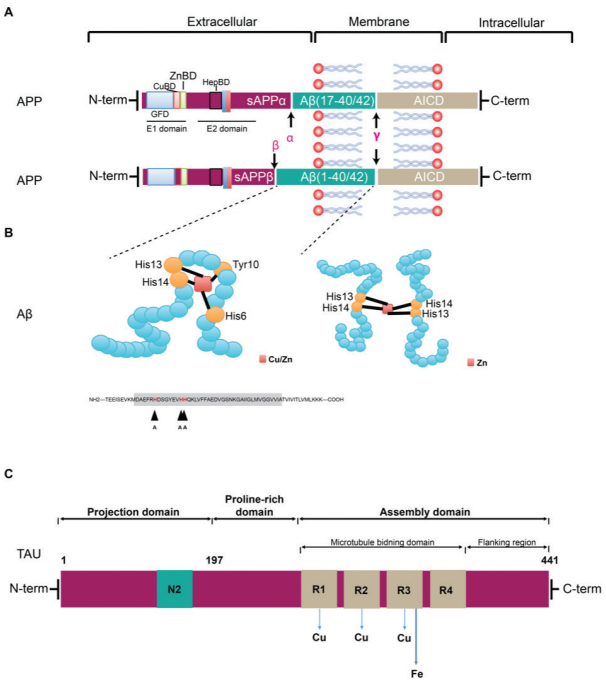

Figure 2 **APP cleavage and APP and tau metal-binding sites.** A) Cleavage of APP by α- and γ-secretases creates the sAPPα and $A\beta_{17\text{-}40/42}$ fragments (upper panel). Cleavage by β- and γ-secretases creates the sAPPβ and $A\beta_{1\text{-}40/42}$ fragments (lower panel). The N-terminal part of APP contains a CuBD, copper-binding domain; ZnBD, zinc-binding domain; HepBD, heparin-binding domain; GFD, growth factor-like domain. **B)** Two of several possibilities of metal interaction with Aβ. Below: Sequence of Aβ, 3 His in the Aβ peptide domain of human wild type (wt) Aβ are important for metal coordination**. C)** Representation of the three main domains studied in tau protein. The picture shows the binding sites of Cu and Fe, respectively, through R1, R2, and R3 regions in the MTB ensuring the binding to the microtubules.

METAL INTERACTIONS WITH AMYLOID-BETA (Aβ)

The Aβ domain of APP is another region that can directly bind Zn and Cu ions; however until now, there is no evidence that this region interacts with metal ions prior to the enzymatic cleavage mediated by α-, β-, and γ- secretases (42) (Figure 2B).

Aggregation of Aβ into insoluble fibrils is a key pathological event in AD and is mediated by the interactions of Aβ with metals, in particular, Zn, Cu, and Fe. Early studies have shown that in particular, the histidine (His) residues in Aβ are responsible for the metal-mediated aggregation of Aβ (43). Interestingly, in mice and rats, the same His residues are not present, which might explain why these animals are more resistant to the amyloid pathology compared to other mammals (44).

The strong chelation properties of Aβ of Zn, Cu, and Fe explain the enrichment of these ions in amyloid plaques and suggest that one potential pathological influence of Aβ might be to sequester metal ions (45) and, through increasing concentrations of redox-active Cu and Fe ions in amyloid plaques, to promote oxidative stress.

Zinc and Amyloid-Beta

Zn is a factor contributing to the neurotoxicity of Aβ through the stabilization of amyloid fibrils (46). Various coordination sites have been proposed for the binding of zinc to Aβ with particular importance of His13 and His6. In vitro studies demonstrated that Zn induces the rapid and extensive aggregation of synthetic Aβ (34, 47), which might act as a seeding factor in the formation of amyloid plaques. In support of these studies, high levels of Zn have been found in the senile plaques of postmortem tissue of AD patients (45) and plaques of genetic AD mouse models. Interestingly, Aβ deposits fail to develop into mature plaques in the cerebellum where vesicular Zn is absent. Scavenging of Zn ions through Aβ may itself be a pathomechanism of AD. A locally decreasing Zn level in the vicinity of plaques contributes to synapse loss (13).

Copper and Amyloid-Beta

Homeostasis is fundamental for all metal ions, but for Cu it is critical because this metal is redox-active and can catalyze and activate O_2, generating reactive oxygen species (ROS) involved in oxidative damage. The soluble monomeric Aβ displays three high-affinity His Cu-binding sites (His6, His13, and His14), which along with the N-terminal amino group and aspartate form a tetragonal complex with Cu ions (48, 49). Cu(II) has been demonstrated to play a crucial role in the formation of β-sheet structures, which are thought to be a preliminary step of the toxic aggregates of the fibrillar form of Aβ. Thus, Cu binding to Aβ has been proposed to play a major role in the neurotoxicity of Aβ. In line with this, a series of studies have reported that Cu chelators rapidly induce the inhibition of Aβ accumulation in transgenic AD mouse models (50, 51).

Other essential trace metals and Amyloid-Beta

Because of Fe^{3+} hydroxide species precipitation, the binding of Fe^{3+} to Aβ seems implausible. Instead, Asp1, Glu3, and the three His residues (His6, His13, and His14) are involved in binding of Fe^{2+}. However, iron mineral deposits in the cortical tissue may occur in vivo and contain magnetite (Fe_3O_4). They have been found in tissue extracted from human AD brain and brain from APP/PS1 transgenic mice (52). The aggregation state of Aβ appears to affect iron redox cycle and consequently may lead to the release of free radicals via Fenton chemistry. Interestingly, the degree of altered iron accumulations in AD is correlated with the amount of Aβ plaque pathology. However, these changes appear to occur after the development of the AD pathological hallmarks (53). Increased aggregation of

Aβ has been observed through the down-regulation of the enzymes that regulate the degradation of extracellular Aβ deposits induced by high Mn levels (54).

Toxic trace metals and Amyloid-Beta

Several studies have suggested that Al interacts with Aβ. Al has been detected in both the Aβ plaques and NFTs. Treatment of neuronal cultures with Al resulted in a marked accumulation of Aβ aggregates in vitro. However, the relevance of this for AD pathology in vivo is currently not well understood (48). Similarly, Hg exposure has been shown to promote the accumulation of Aβ deposits in vitro. Cd, like Mn, has been reported to reduce the expression of Aβ-degrading enzymes, resulting in an increased Aβ accumulation(48).

METAL INTERACTIONS WITH TAU

In AD, Tau aggregates due to hyperphosphorylation, abnormal splicing, or mutation in the tau encoding gene (55, 56). AD is the most common tauopathy among degenerative brain diseases. The tauprotein, with a molecular weight between 50 and 68 kDa, is encoded by a single gene (*MAPT*) located on chromosome 17q21 in humans. Tau is mainly localized in neuronal axons, but also dendrites (57), in the central nervous system (CNS). Tau is a microtubule-associated protein (MAP) implicated in the stabilization and integrity of microtubules (MT) in neurons, and its activity is regulated through a phosphorylation-dependent mechanism (58). In physiological conditions, tau is phosphorylated (facilitating the disassociation of the protein from the MT) and dephosphorylated (promoting the binding with MT) through the activity of tau kinases and phosphatases (59). In the human brain, under developmental control, six different isoforms of tau exist with a variation in size from 352 to 441 amino acids. The isoforms differ in the inclusion or exclusion of N repeats (0N or 1N or 2N) at the amino-terminal region and for the presence of three (3R) or four (4R) MT-binding domain (MTB) repeats (R) in the carboxyl-terminal part of the molecule (60).

The major domains identified in the tau protein are the *projection domain*, situated in the acidic N-terminal part, and the *assembly domain,* localized in the basic C-terminal domain. The two domains with the opposite charge are separated from one another by the *proline (Pro)-rich region,* situated in the middle part of the protein. Here, tau interacts with proteins containing an SH3 domain. Further, the Pro-rich region is the target of different Pro-directed kinases and also FYN-tyrosine kinases (61). The assembly domain, through R1–R4 repeat regions and flanking domain, binds microtubules and supports their assembly. This domain is the key in the regulation of the phosphorylation state of the tau protein (62). On the contrary, the projection part does not interact with microtubules but projects away from their surface interacting with other cytoskeletal elements, mitochondria or the neuronal plasma membrane (63, 64).

Phosphorylation of tau plays a crucial role in the pathogenesis of AD (65, 66) introducing negative charge(s) that promote an electrostatic interaction with metal ions (67) (Figure 2C). Tau abnormal phosphorylation leads to an abnormal structure, that is, polymerized into paired helical filaments (PHFs) which may further

aggregate to form NFTs, assuming the shape of a toxic protein deprived of the biological functions typical of the MAP family. It was reported that in AD brains, NFTs include metals, confirming an association between endogenous redox-active transition metals and metal-binding sites in tau (68). Binding of a series of metal ions including the essential biometals, Zn, Cu, Fe, Mg, and Mn, and non-essential trace metals, Pb, Cd, Hg, and Al, may promote tau hyperphosphorylation and induce tau aggregation. In contrast, Fe and lithium (Li) reduce the abnormal phosphorylation of tau (48). For this reason, biometal homeostasis is essential, and the disruption of this balance may play a key role in the pathogenesis of AD.

Zinc and Tau

A disruption in the Zn homeostasis leads to a series of pathogenic conditions in the AD brain, including the formation of NFTs composed of hyperphosphorylated tau. Recent studies show that Zn is involved in the mechanism of tau hyperphosphorylation via two different interactions: in vitro, Zn can directly affect tau at serine (Ser) and Pro sites, at threonine (Thr) and Pro sites or via two Cys residues: C291 and C322 (69). At the same time, Zn can indirectly hyperphosphorylate tau protein, by activating kinase and phosphatase pathways, for example activating Raf/mitogen protein kinase and inhibiting phosphatases such as PP2A (70, 71).

These two independent ways of action have different effects on tau toxicity. It has been demonstrated that the direct interaction between tau and Zn plays an important role in tau toxicity: after removing the Zn-binding site, tau toxicity is completely abolished, assuming that the toxic effect of tau necessitates both the presence of hyperphosphorylation and Zn bond. Tau hyperphosphorylation pathways appear to be less toxic, compared to tau toxicity that occurs from the direct binding between tau and Zn (72). Recently, it has been discovered that Zn could be considered a catalyst, accelerating the aggregation of tau-R3 complexes and, at the same time, promoting the formation of tau oligomers (73, 74). Thus, correct Zn homeostasis in AD is fundamental because abnormally high concentrations of this mineral induce the development of granular tau aggregates, while abnormally low concentrations of Zn lead to amyloid fibril formation (75).

Copper and Tau

A high concentration of Cu (0.4 mM) was reported in amyloid plaques and NFTs. Thereby, NFT may be linked to high levels of redox-active Cu (68). Besides, Cu is involved in tau hyperphosphorylation by activating the cyclin-dependent kinase (CDK)5/p25 complex. Tau hyperphosphorylation resulting from the activation of GSK-3β kinase by Cu is controversial: some studies suggest that GSK-3β kinase is activated by Cu (76, 77), while other studies propose that GSK-3β kinase may not necessarily be involved in the abnormal phosphorylation of the protein (78). The binding between tau and Cu is highly selective. Studies revealed that the full-length Human Tau40 isoform (K32) can bind one Cu for each monomer (1:1 binding stoichiometry) with a dissociation constant (K_d) close to 1 μM via two Cys residues. The sequences mediating the binding of Cu are ^{287}VQSKCGS^{293} and ^{310}YKPVDLSKVTSKCGS^{324}. An analysis conducted by circular dichroism and nuclear magnetic resonance (NMR) spectroscopy showed only limited formation

of aggregates after binding Cu because the addition of Cu to K32 does not affect the secondary structure, and thus, tau remains mostly disordered (79). In vitro, it has been demonstrated that Cu can bind different tau fragments containing diverse MTBR such as R1 and R2, showing alterations in the secondary structure (80, 81). Furthermore, the interactions between tau R2 and Cu lead to the production of H_2O_2 (82). The repeat R3 can be associated with more than one Cu ion via two His residues (83).

The role of Cu-binding to tau remains controversial, although some studies suggest that the binding between Cu and tau inhibits the formation of abnormal aggregates in vitro (78, 81). For example, increasing intracellular Cu levels by the addition of Cu–bis (thiosemicarbazone) complexes, inhibits tau hyperphosphorylation (76).

Iron and Tau

Fe dysregulation is linked to oxidative stress in tauopathies. Fe, as Zn and Cu, interacts with some of the isoforms of the tau protein, causing irreversible structural changes. The result of this interaction is protein aggregation and/or oxidative stress, through the Fenton reaction, perpetuating a condition of cellular damage. Analysis of postmortem AD brains shows increased Fe levels in several brain regions (84).

In the human body, Fe is available in two oxidation states: Fe^{3+} (redox-inert state) that is stored in ferritin and Fe^{2+} (redox-active). The iron status associated with NFTs in AD is Fe^{3+}, which can induce the aggregation of hyperphosphorylated tau. Fe-binding sites using His residues have been identified in tau (85). The hyperphosphorylated status of tau may not involve Fe^{3+} interacting with the protein, but Thr phosphorylation can regulate the interaction between tau and Fe^{2+} (86). Thus, the phosphorylation level of tau causes conformational changes of tau to mediate tau–Fe interactions (87). In addition to a direct interaction, Fe induces tau hyperphosphorylation, both in vitro and in vivo, by activating the CDK5/p25 complex and GSK-3β and MAP kinases (88). This evidence suggests a possible role of iron involved as a co-factor for tau aggregation.

Other essential trace metals and Tau

In AD patients, Mg levels appear lower (540–625 μg/g) compared to the physiologic range (620–680 μg/g) (89). In vivo, data obtained from an AD transgenic mouse model show that Mg increases the phosphorylation of the GSK-3β kinase at Ser9, which in turn reduces the hyperphosphorylation of tau protein (90). Additionally, in postmortem brains of patients affected by AD, the level of Mn appears to be higher (91). An increase of Mn levels is related to abnormal tau aggregation and its hyperphosphorylation, mediated by GSK-3β kinase (92).

Toxic trace metals and Tau

Al is the most widely exogenous metal ion distributed in the environment. As Fe^{3+}, Al^{3+} is a trivalent cation that influences protein phosphorylation of tau (93, 94). Recent data show that Al can promote the formation of sodium dodecyl

sulfate (SDS)–resistant tau oligomers after tau phosphorylation (95). The role of Al in AD has been intensively investigated since NFT-like deposits were discovered in mammalian brains after intracerebral Al injection (96). Al has been shown promoting tau aggregation through the down-regulation of PP2A activity and an increase of CDK5 and GSK-3β kinase levels (97). Thus, Al, although not directly binding to tau, may have a role as co-factor in AD (98).

Further, heavy metals such as Cd, Pb, and Hg have been implicated in AD pathology (99). Data show that Cd is involved in the formation of NFTs (100, 101). Both in cell models and in in vivo studies, Cd increases the activation of GSK-3β kinase, causing the hyperphosphorylation of tau (102). Similarly, Pb has been reported to modulate tau aggregation by increasing the activity of CDK5/p25 complex and GSK-3β kinase (103). Hg was demonstrated to inhibit tubulin that has a very high-affinity binding-site for Hg (104). Once Hg binds tubulin, the structural integrity of the protein is impaired. The final result of this interaction between Hg and tubulin is the formation of NFTs (105). Also, Hg is involved in tau hyperphosphorylation; the mechanism starts with the oxidative stress induced by Hg, ultimately affecting tau phosphorylation status (106).

OTHER METAL-BINDING PROTEINS AND THEIR ROLE IN AD

Several of the effects of an altered trace metal status in AD such as increased oxidative stress, neuroinflammation, and effects on synapses are mediated by excess or lack of trace metals for binding to proteins other than Aβ and tau. Together with several other factors in AD, oxidative stress leads to an activation of the immune system. The immune system is highly dependent on trace metal biology. Especially, Zn signaling seems to be a key mediator of inflammatory responses. For example, the activity of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), a major regulator of pro-inflammatory cytokines such as interleukins (IL) (107), is regulated, among others, by Zn through Zn binding of the IKK (IκB kinase) complex member IKKβ (108). Further, the formation of senile plaques made of Aβ aggregates stimulates inflammasomes, such as the NLRP3 (nucleotide-binding domain and leucine-rich repeat-containing family, pyrin domain-containing-3) inflammasome that detects the inflammatory Aβ aggregates and responds by forming active IL-1β through secreting caspase-1 (Casp-1) (109). IL-1β acts as an inflammatory cytokine (110), which leads to the creation of an inflammatory environment around the plaque. This ultimately decreases plaque degradation and destruction by microglia cells. Zn deficiency and/or high Cu levels facilitate NLRP3 inflammasome activation (111) and thereby the production of IL-1β in macrophages (112).

Initially, Pro-IL-1β is expressed in response to damage-associated molecular patterns (DAMPs) that bind to pattern recognition receptors (PRRs) on the macrophage to upregulate pro-inflammatory gene expression. Inflammation, protein misfolding, and aggregation, as well as neurodegeneration, lead to increased levels of so-called alarmins or DAMPs that include several cytokines including those from the S100 family. The S100 proteins are engaged in classical calcium-activated signaling but recent work has shown their involvement in new biochemical mechanisms in the brain related to the prevention of protein aggregation (113) and

sensing of neuronal Ca and Zn levels (114). Therefore, S100 alarmins are implicated in the maintenance of protein homeostasis (proteostasis) and metal ion homeostasis (metallostasis) in the brain. Upon activation and at high (μM) concentrations, S100 proteins act as extracellular cytokines via RAGE (receptor for advanced glycation end-products) mediated signaling. RAGE persistent engagement increases S100 extracellular levels via NF-κB activation resulting in a positive feedback cycle (115). Glial S100B and S100A9 proteins show increased expression in response to several risk factors for AD, including aging (116). Interestingly, S100B undergoes metal-binding-induced conformational changes and thereby delays the onset of Aβ aggregation by interacting with $A\beta_{1-42}$ monomers inhibiting primary nucleation (113). However, high levels of S100B can elicit alterations in intracellular Zn concentrations (114).

The increase in S100B proteins and accumulation of Aβ as a factor for trace metal imbalances also has direct effects on Zn signaling at excitatory glutamatergic synapses. It has been shown that the dynamics of major postsynaptic scaffold proteins of these synapses (SHANK2 and SHANK3) are dependent on Zn availability (117). Studies have shown that SHANK platform disassembly is linked to the molecular pathology of AD (118, 119), and recent research confirmed that the progressive accumulation of Aβ results in decreased Zn concentrations at the synapse, which in turn leads to disruption of SHANK3 scaffold formation, and ultimately, loss of synapses (13). Thus, Zn sequestration by protein aggregates in AD may be a contributor to the cognitive impairments caused by the loss of synapses through trapping synaptic Zn rather than through neurodegeneration in general (120). In addition, NMDA receptors at synapses are Zn-binding proteins (121). Increased trapping of Zn lowers the inhibitory activity of Zn on the NMDAR. Excessive stimulation of receptors at the excitatory synapse has been linked to neuronal death through excitotoxicity leading to chronic neurodegeneration in AD (122). Together, these metal-imbalance-driven signaling pathways create a vicious cycle leading to increased inflammation, oxidative stress, and neuronal damage (Figure 3).

METAL DETECTION FOR AD DIAGNOSIS

Several metal bioimaging strategies have been developed not only to examine the distribution of metals in human clinical AD brain tissue and AD mouse models but also to diagnose and monitor the progression of AD (123). In the clinical setting, the most common imaging tool is magnetic resonance imaging (MRI). This technique focuses on Fe due to its magnetic properties and its abundance in the brain. The latest MRI technology provides sufficient resolution to detect regional differences and has the major advantage that it can be applied to living patients rather than being a tool for postmortem analysis only (124). The presence of localized Fe can be detected by MRI (T2). However, although detecting general metal dyshomeostasis, MRI has limitations in visualizing metal-loaded plaques directly with high resolution. Further development of metal-based compounds or compounds visualizing metal homeostasis, such as a Cu-64-labelled–*bis* (thiosemicarbazonato) complex for clinical application in positron emission tomography (PET), and improvement of imaging devices may lead to more precise

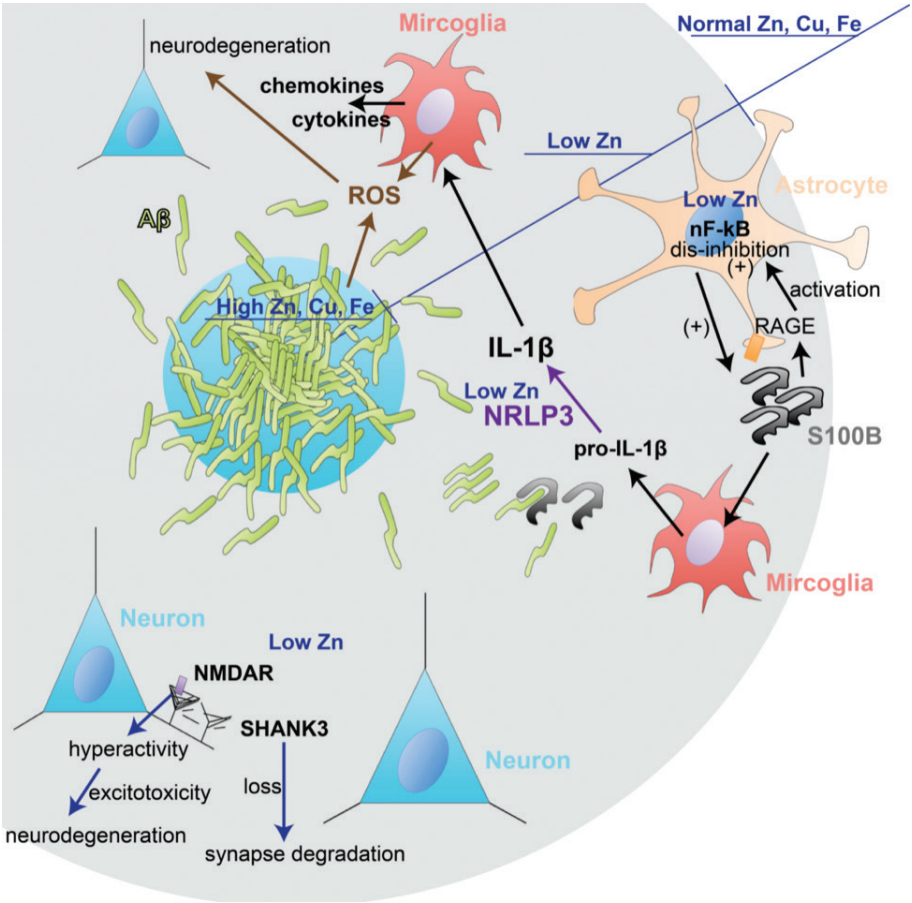

Figure 3 **Interaction between Aβ plaques, glial cells and trace metals.** Senile plaques sequester large amounts of Zn, Cu, and Fe, creating a zone of metal depletion, especially Zn depletion, in their vicinity. While high levels of Cu and Fe at the center of plaques may contribute to the generation of ROS and damage neurons through oxidative stress, Zn deficiency in a zone surrounding plaques will lead to further effects: Accumulation of Aβ aggregates leads to the release of S100B from astrocytes. S100B as DAMP can initially prevent Aβ aggregation. However, S100B signals back to astrocytes via RAGE receptor activation that will, in turn, activate NF-κB. Active NF-κB is dis-inhibited by low levels of Zn and thus results in further production of S100B, which enters a positive feedback cycle. High levels of S100B further deplete Zn through Zn binding. In response to high DAMP levels (S100B), microglia cells will produce pro-IL-1β. This will be cleaved by Casp-1-dependent processes through the NLRP3 inflammasome. NLRP3 activity is further increased by low Zn levels. Production of IL-1β leads to further generation of ROS and release of cytokines and chemokines from microglia cells that facilitate NFT formation and neurodegeneration. Further, low Zn levels facilitate excitotoxicity through dis-inhibition of NMDAR signaling, and low levels of Zn destabilize the postsynaptic Shank3 scaffold resulting in synapse loss.

diagnosis and monitoring of progression and therapeutic effects based on the role of trace metals in AD in the future (124).

METALS HOMEOSTASIS AS A THERAPEUTIC STRATEGY FOR AD

Based on the interactions of metals with several key proteins of the AD pathology, different therapeutic approaches aimed at restoring or manipulating metal homeostasis and, thereby, regulating oxidative stress, tau phosphorylation, Aβ aggregation, and inflammation have been developed in the last decade.

For example, metallothionein 3 (MT-3), a key regulator of metal homeostasis in neural tissue, has been found down-regulated by up to 30% in AD brains. Given that MT-3 contributes, among others, to detoxification and storage of heavy metals, regulation of Cu and Zn metabolism, and modulation of Aβ endocytosis of astrocytes (125), increasing MT-3 levels in AD has been explored as therapeutic strategy. In vivo studies demonstrated that effects of Zn-loaded MT-3 treatment in a mouse model for AD (Tg2576 mice) are inconsistent if MT-3 is injected subcutaneously. However, MT-3 injected intracerebroventricularly is able to ameliorate behavioral deficits and hippocampal impairments in APP/PS1 mice. In these mice, MT-3 treatment was also able to restore metal homeostasis, inhibit Aβ aggregation, and reduce oxidative stress and neurodegeneration (125).

Another interesting treatment strategy is based on metal protein attenuating compounds (MPACs): Clioquinol (CQ) represents the prototypic MPAC. It is a small hydrophobic molecule that can cross the BBB and that has moderate affinity for metal ions. When administered to Tg2576 mice, a 49% decrease of Aβ in the brain of AD model mice compared to control mice was shown (126). In humans, oral CQ treatment for 36 weeks of severely affected AD patients was able to significantly prevent cognitive deterioration. Subsequent clinical studies of this compound were not pursued. However, PBT2, a highly soluble derivate of CQ (a second-generation MPAC), has been used first in APP/PS1 mice and then in human clinical trials (phase I and II). The results showed improved cognitive performance and reduced Aβ load in the mouse model. A 12-week-long treatment of 78 patients with early AD showed that PBT2 is safe (127). Although the effects of PBT2 were inconsistent, executive dysfunction was significantly reduced in the patients. Several other metal chelators were engineered over the last years, and most of them are currently investigated for use in AD. Some of them have been shown to be effective at inhibiting Aβ–metal interactions both in vitro and in vivo. For example, it has been demonstrated that the normally insoluble Aβ deposits of postmortem brain tissue from AD patients can be solubilized in aqueous media in the presence of specific Cu chelators (128).

Another promising approach is the delivery of metals directly to the brain using nanotechnological approaches. Polymeric g7-poly-lactide-co-glycolide (PLGA) nanoparticles (NPs) are able to cross the BBB and release metals within the brain. This system has been considered as a Trojan horse strategy to effectively deliver Zn to the brain with a low-toxicity profile (129). Three hours after ip injection of NPs, an increase of Zn levels in the brain and the increase of zinc-sensitive

genes such as MT and Zn transporters were seen (130). The same pharmacological approach applied to APP23 mice, an animal model of AD, showed promising effects such as Aβ dis-aggregation, a reduction of inflammation, and synapse stabilization (129). Thus, both redistribution of metals bound to Aβ through MPACs and increase in metal levels that has dropped through trapping of metals in Aβ deposits have beneficial effects. However, additional research is necessary to redefine time point of application, duration, and concentration of NP-based metal delivery.

CONCLUSIONS AND FUTURE PERSPECTIVES

In general, impaired biometal homeostasis and/or the accumulation of non-essential trace metals have significant effects, most prominently on proteotoxic stress, synapse function, oxidative stress, and inflammatory processes. Building on the metal-binding abilities of key proteins in AD, brain imaging-based methods for the diagnosis of AD in humans have been, and are, currently developed. However, despite improving techniques for the detection of trace metals in the brain, re-establishing metal balances remains a difficult task. Initial studies using Zn ionophores have been promising and showed that targeting metal homeostasis in AD may be one of the most auspicious therapeutic strategies. However, new targeted and improved approaches are needed in the future.

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