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A Potential Role for Zinc Alterations in the Pathogenesis of Alzheimer's Disease

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Abstract

Alzheimer's disease (AD), one of the major causes of disability and mortality in Western societies, is a progressive age-related neurodegenerative disorder. Increasing evidence suggests the etiology of AD may involve disruptions of zinc (Zn) homeostasis. This review discusses current evidence supporting a potential role of Zn and zinc transporters (ZnTs) in processing of the amyloid beta protein precursor (APP) and amyloid beta (Aβ) peptide generation and aggregation.

Keywords

Alzheimer's disease; amyloid beta peptide; preclinical Alzheimer's disease; zinc; zinc transporter proteins; mild clinical impairment

Overview of Alzheimer's disease

At the beginning of the twenty-first century, Alzheimer's disease (AD) is the most common form of adult-onset dementia, and currently affects 4.5 million Americans (1) and may affect 13 million by 2050, unless preventive strategies are found. AD is the eighth leading cause of death in the United States (2) with direct and indirect medical and social costs in excess of $100 billion per year (3). Currently about 6% of the population over age 65 suffers from AD and incidence rates increase with age (4).

The symptoms of AD progress from mild memory loss to profound dementia. In general, AD patients die from secondary infections and illnesses (4). Data from the Baltimore Longitudinal Study of Aging suggest that median survival times following diagnosis of AD depend on the patient's age at diagnosis and ranges from 8.3 years for subjects diagnosed at age 65 years to 3.4 years for those diagnosed as having AD at age 90 (5). Clinically, AD represents a chronic progressive neurodegenerative disorder characterized by three primary groups of symptoms: (i) cognitive dysfunction, (ii) non-cognitive symptoms, and (iii) difficulties in performance of activities of daily living (ADL) (4).

Gross examination of the AD brain typically shows marked atrophy, with widened sulci and shrinkage of the gyri (3). Neuropathologically, the AD brain demonstrates selective neuronal and synapse loss, particularly in the hippocampus, amygdala, entorhinal cortex, neocortex and nucleus basalis of Meynert. In addition, there is an abundance of senile plaques (SP)

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composed of amyloid beta peptide (Aβ), a product of amyloid precursor peptide (APP) proteolytic cleavage, and neurofibrillary tangles (NFT) containing hyperphosphorylated tau. SPs are classified as i) diffuse plaques, that are made of extracellular amorphous Aβ deposits without neurites; and ii) neuritic plaques, composed of extracellular deposits of insoluble Aβ surrounded by dystrophic neurites, activated microglia and reactive astrocytes (reviewed by Markesbery and Lovell, 2006).

There is a considerable interest in understanding how AD progresses and in the definition of the individual stages of the disease. Based on clinical examination and histopathological analysis of postmortem brain, diagnosis of early stage AD (EAD) and late-stage AD (LAD) is possible (3). The age when initial clinical and neuropathological events trigger AD is a debatable issue. Although clinical manifestations of AD are age-dependent, increasing evidence suggests the initial neuropathological events that trigger AD may begin at an earlier age (6,7). Data from a neuropathological study of 2,661 brains (age range from 25 to 95 years) show neurofibrillary alterations develop at around 40 years of age (8). Another study showed amyloid plaques in the neocortex distinguish early stages of AD from normal brain aging (9).

In the early 1990's, the concept of mild cognitive impairment (MCI) as an amnesic state with a high rate of progression to AD was introduced (10). Clinically, amnesic MCI patients have more pronounced memory impairment than would be expected at their age but without the notable impairment observed in defined ADLs (11). Longitudinal studies suggest that cognitive impairments in this early stage may remain relatively constant for several years (11). The stable phase of MCI ends with a detectable decline in cognitive function, lasting two to five years (12). Prospective studies have shown that people with amnesic MCI are fifteen times more likely to have developed dementia at follow-up, suggesting it maybe a precursor to AD (4).

In addition to the concept of amnesic MCI as an early stage of AD, a preclinical stage of AD (PCAD) has recently been proposed that is characterized by AD neuropathology in the absence of clinical manifestations and may precede the development of MCI and AD (6,13,14). An understanding of the pathological processes that trigger development of each stage of AD would be beneficial in prevention and treatment of the disease.

The cause of AD is unknown, but numerous studies have linked several risk factors with the disease (3,4). Age is considered to be the main risk factor for AD (3). The second most important risk factor for AD is a positive family history (3). The risk for first degree relatives of people with the disease is 10–40% higher than in unrelated people (4). Other possible risk factors for AD include gender (15), low levels of education (15,16), low linguistic ability in early life (15–17), head injury (18,19), hypertension (20), diabetes (21) and depression (22). Three genes are responsible for more than 90% of early onset familial cases: presenilin-1 (PS1) located on chromosome 14, presenilin-2 (PS2) on chromosome 1, APP on chromosome 21 (23). Apolipoprotein E (ApoE) is a susceptibility factor for AD (24). ApoE is involved in cholesterol transport and probably neuronal repair, and exists as three alleles: ε2, ε 3, and ε4 (3). The ε3 isoform is the most common and the ε2 isoform the least common in the general population. The presence of the ε4 isoform increases the risk of developing AD, and the ε2 allele appears to decrease the risk (24).

Although the etiology of AD remains enigmatic, it is plausible that multiple triggers contribute to AD. Several hypotheses have been proposed for the etiology/pathogenesis of AD including the oxidative stress hypothesis (25), the amyloid hypothesis (3), and the toxic trace metal hypothesis (26) among others. Trace elements play an important role in metabolism (27) and roles for trace metals has been proposed for the pathogenesis of

neurological disorders such as Wilson's disease and Parkinson's disease (28). Recent studies suggest disruptions of trace elements are involved in the neuropathology of AD (26,29,30).

Proteins which regulate zinc homeostasis on brain

In the past few decades, a variety of structural, catalytic and regulatory functions of zinc (Zn) have been described and Zn is often called "the calcium of the twenty-first century" (29). Zn is the second most abundant trace element (concentration of μg/g of wet tissue) in the body after iron (31). Crucial decisions about cellular growth, proliferation, differentiation and programmed cell death involve Zn in ionic or protein-bound forms (26). Zn moderates the activity of at least 300 enzymes and transcription factors and plays an important role in DNA and RNA transcription and replication (32). Zn is also involved at all levels of signal transduction in mammalian cells (33).

The mammalian brain contains ~10–20 μM Zn (34). Zn concentrations in gray matter vary from 150 to 200 μM (35). The concentration of free Zn ions in the extracellular space of healthy brain tissue is in the range of 1 to 10 nM (29), and in intracellular vesicles Zn is in the mM range. During neurotransmission, concentrations of Zn can reach values from 0.5 μM at the basal level (36) to 300 μM in synaptic cleft (37).

In neurons, Zn stabilizes glutamate in synaptic vesicles and modulates the behavior of postsynaptic membrane receptors and ion channels (38). Significant concentrations of glutamate-and Zn-releasing terminals in neocortex and amygdala suggest Zn may be involved in the process of learning and formation of memory. It has been suggested that Zn is the key factor of both developmental and experiential neuroplasticity (29). Studies by Takeda et al. demonstrate that rats maintained on a Zn deficient diet for four weeks did not have significant changes in Zn levels in hippocampus compared to controls (39), suggesting that Zn levels in mammalian brain are tightly regulated and remain at the same level during relatively short periods of Zn deficiencies. In addition, studies by Chowanadisai et al. showed Zn deficiency caused depleted plasma Zn but not brain Zn concentration in neonatal rats (40).

There are three distinct pools of Zn in the brain: a protein membrane complex pool or membrane bound metalloprotein pool, an ionic or chelatable pool of free or loosely bound ions and a vesicular pool which is released during neurotransmission (34). Chelatable Zn is present in the hippocampus, amygdala, visual, and somatosensory cortex (31).

Three major classes of proteins regulate Zn at the cellular level: metallothioneins (MT), Zn transporter (ZnT) proteins, and members of zinc-regulated and iron-regulated transporter proteins (ZIP) (41). MTs coordinate intracellular Zn trafficking. ZnTs mediate Zn efflux from cells or influx into intracellular vesicles whereas ZIPs promote Zn transport from extracellular space or from intracellular vesicles to cytosol.

MTs are relatively small 6–7 kDa cysteine-rich proteins (41). Spatial arrangement of ~ 20 cysteines in one MT molecule accounts for its high affinity metal binding ($K_{Zn} = 3.2 \times 10^{-13}$ M^{-1}, pH 7.4) (42). The role of MTs is to protect cells from Zn deficiency or toxicity (43). MTs are ubiquitously expressed and their levels are particularly high in parenchymal cells of the intestine, pancreas, kidney, and liver (41). To date, four MTs have been characterized. MT I and MT II are present in most mammalian organs including the brain (43). MT III was found predominantly in the brain (44), whereas MT IV is in epithelial cells (45). Numerous reports describe Zn-dependent expression of MTs and their responses to oxidants, cytokines, and glucocorticoids (reviewed in Cousins et al., 2006).

ZIPs function to increase intracellular concentrations of Zn by importing Zn inside the cell from the extracellular space or by releasing Zn from intracellular organelle storage when cytosolic Zn concentrations are low. The mammalian ZIP family consists of 14 members (41). ZIPs are predicted to contain eight transmembrane domains with a histidine rich loop between the third and fourth domains. The mechanism of ZIP-mediated transport is not well understood, but studies suggest that it could be a facilitated process driven by a concentration gradient because activities of human ZIP1 and ZIP2 do not require ATP nor K^+ or Na^+ gradients. (46,47). Mammalian ZIPs are found in various organs. mRNA of ZIP1 is ubiquitously expressed (47), whereas ZIP2 is present only in the spleen, small intestine, bone marrow and in blood cells (46,48,49). ZIP3 expression is also high in the spleen and bone marrow (46), whereas ZIP4 expression was found in the small intestine and kidney (50). A mutation in the human ZIP4 gene, responsible for intestinal absorption of zinc, has been discovered in the Zn metabolism disorder acrodermatitis enteropathica (50). Human ZIP5 expression is high in the intestines, liver, kidney, and pancreas (51) whereas ZIP6 is expressed in the prostate and placenta (52). Structural evidence suggests a number of other mammalian genes encode proteins of the ZIP family most of which have been identified using mouse and human sequence analysis but have not yet been characterized (53).

Sequestration of Zn from the cytosol of the cell to the extracellular space or intracellular compartments is regulated by Zn transporters (ZnTs). In 1995 Palmiter and Findley discovered the first mammalian Zn transporter (ZnT-1) (54) with an additional 9 ZnT's described since. Most ZnTs have six transmembrane domains and histidine rich loops which connect these domains (55). Four ZnTs are present in mammalian brain: ZnT-1, ZnT-3, ZnT-4, and ZnT-6 and recently we detected the fifth, ZnT-2, in human brain (unpublished results).

Immunohistocytochemistry shows ZnT-1 is located at the plasma membrane and is responsible for Zn export from the cytosol to the extracellular space during periods of elevated cytosolic Zn (Palmiter and Findley, 1995). ZnT-1 can be regulated by dietary Zn (53). This transporter is abundant in areas rich in synaptic Zn and has been proposed to have a protective role against Zn cytotoxicity in the nervous system (56). Our previous study of ZnT-1 showed a significant decrease in hippocampus and parahippocampal gyrus (HPG) of MCI, but a significant elevation in HPG of early EAD and LAD subjects (57).

The exact mechanism of Zn transport by ZnTs is not known. Palmiter and Findley observed the rate of Zn efflux increased as extracellular Zn increased, and suggested Zn efflux mediated by ZnT-1 is an energy dependent transport, arguing against ZnT-1 being a channel or facilitated transporter (54). In contrast, Takeda et al. reported rats on a Zn deficient diet had increased levels of Zn in the brain (58), supporting the idea proposed by Chowanadisai et al. that low systemic Zn may lead to decreased ZnT-1 levels to maintain Zn brain levels (40). Studies from Sekler's group suggest ZnT-1 reduces Zn influx through L-type calcium channels without increasing Zn efflux, thus ZnT-1has a dual role: regulation of calcium influx, and attenuation of Zn permeation and toxicity in neurons and other cell types (59).

In 1996 Palmiter and colleagues isolated ZnT-2 from a rat kidney cDNA expression library and characterized this transporter (60). They introduced ZnT-2 into Zn-sensitive baby hamster kidney cells (BHK) and showed that cells expressing ZnT-2 accumulate Zn in endosomal compartments (by staining with acridine orange or LysoTracker) in the presence of excessive extracellular Zn. Therefore, it was concluded that ZnT-2 can protect against Zn toxicity by facilitating Zn transport into endosomal and lysosomal compartments (60). A Zn deficient diet caused depletion of ZnT-2 levels in mammary gland of rats for both mRNA and protein, whereas Zn intake brought ZnT-2 expression back to normal (61). The predicted structure of ZnT-2 is similar to ZnT-1, but the amino-acid homology of ZnT-1 and

ZnT-2 is only 26% (41). Although ZnT-2 mRNA is barely detectable in mouse brain by reverse transcriptase polymerase chain reaction (RT-PCR) analysis (60,62), we detected mRNA of ZnT-2 in human brain and also detected significant changes of ZnT-2 protein levels in AD brains compared to normal control (NC) subjects (unpublished results).

In 1996 murine ZnT-3 gene was cloned by virtue of its homology to the ZnT-2 gene (62) and was detected in brain and testis (60). It was also found that inactivation of ZnT-3 prevents the accumulation of zinc in synaptic vesicles. Expression of ZnT-3 in a zinc-sensitive cell type did not result in a more zinc-resistant phenotype in contrast to the phenotypic alterations induced by ZnT-1 and ZnT-2 expression (63). A potential role for ZnT-3 and synaptic Zn in SP formation in transgenic mice was investigated in studies that showed crossing mice expressing mutant APP with ZnT-3 null mice led to diminished Aβ deposition (64). In addition, Friedlich and colleagues (65) found that ZnT-3 knock-out mice also showed reduced cerebral amyloid angiopathy. Thus, synaptic ZnT-3 activity may promote cerebral amyloid angiopathy by raising exchangeable Zn^{2+} concentrations in the perivascular spaces of the mouse brain. In our studies of LAD and control brain, we find no significant alterations of ZnT-3 in LAD.

ZnT-4 is located in lysosomal or/and endosomal compartments and functions to sequester Zn in these compartments (66). ZnT-4 shares 67% and 62% homology with ZnT-2 and ZnT-3 respectively (63). ZnT-4 confers Zn resistance to Zn-sensitive yeast, and a single point mutation in the ZnT-4 gene causes inherited Zn deficiency in the lethal milk mouse (66). Lethal mouse syndrome is characterized by the inability of pups to survive before weaning. The lethal genotype is the result of decreased Zn transport from the mammary gland of lethal mouse dams to their milk (63). In our study of human brain we detected elevated levels of ZnT-4 protein in HPG of EAD and LAD subjects (67).

Yamaguchi-Iwai et al. identified a ZnT-5 in 2002 (68). Human ZnT-5 cDNA codes for a protein with 15 predicted membrane-spanning domains. ZnT-5 is ubiquitously expressed in all tested human tissues and abundantly expressed in the pancreas suggesting that ZnT-5 plays an important role for transporting zinc into secretory granules in pancreatic β-cells (66).

ZnT-6 functions to sequester Zn in the trans-Golgi network (TGN) as evidenced by overlapping of ZnT-6 antibody staining with staining for TGN38 and transferrin receptor in the normal rat kidney cells (69). In contrast to other ZnTs, ZnT-6 has multiple serine residues replacing histidines in the loop region. Gitschier's group proposed that serine may coordinate Zn binding with the histidine residue at the C-terminal end of ZnT-6 to facilitate Zn trafficking across the membrane (69). Intracellular distributions of both ZnT-6 and ZnT-4 are regulated by Zn in the normal rat kidney cells. In our previous study we found levels of ZnT-6 increased in HPG and superior and middle temporal gyrus (SMTG) of LAD and HPG of EAD subjects compared to NC subjects (70). In our study of PCAD brain we found an increase of ZnT-6 in PCAD HPG and cerebellum (CER) compared to NC subjects (71). Confocal microscopy experiments revealed that locations of ZnT-6 in PCAD and MCI brain correlate with the sites of early formation of NFTs (70,71).

Furthermore, increased levels of ZnT-6 were associated with fragmentation of Golgi apparatus, suggesting the possible role of ZnT-6 in neurodegeneration. Increased ZnT-6 in degenerating neurons could cause Zn accumulation in the TGN, and could alter normal sorting and trafficking of proteins and lipids and contribute to degeneration of neurons and production of cytoplasmic lesions such as NFTs (70).

Similar to ZnT-6, ZnT-7 sequesters Zn in the TGN but has expression restricted to small intestine and lung (72). The ZnT-8 protein is expressed in pancreatic β-cells and thus may

be of primary importance for the insulin secretory pathway (73,74). Although more recent studies demonstrate ZnT-8 expression in lymphocytes (75) there is limited expression in murine brain. brain. In recent studies Kelleher et al. described increased levels of ZnT-9 in mammary cells during lactation (76) and while Seve et al. used *in silico* approaches and analysis of genomic databanks to identify the full length sequence of ZnT-10 which has high amino acid sequence homology to ZnT-1 (77).

Zinc Toxicity in Alzheimer's disease

Although Zn is a redox-inactive metal and is considered relatively non-toxic, an increasing body of evidence indicates that free ionic Zn is potentially damaging to neurons. Cell culture studies by Yokoyama et al. showed 15 min exposure to 300–600 μM Zn caused extensive cortical neuron death (78). Ducray et al. used intranasal perfusion of $ZnSO_4$ in one month old and six month old mice and showed a total destruction of olfactory neurons in a few days (79).

Exact mechanisms of Zn mediated cell death are unclear, but numerous hypothetical mechanisms have been proposed (30,38). The dominant theory of Zn accumulation and neurotoxicity has been the hypothesis of Zn translocation (30,38). This theory accentuates the role of Zn that is released from presynaptic vesicles, crosses the postsynaptic membrane via channels or transporters (translocation) and causes neuronal death. Recent studies of Zn transport and function support the translocation theory of Zn (29). Sensi and colleagues found that Zn ions may replace calcium ions and serve as a substrate for Na^+-Ca^{2+} exchanger. They also hypothesized the existence of Na^+-Zn^{2+} channels (80). Other studies suggest Zn^{2+} may act as a neurotransmitter and mediate apoptosis (30). Recently, the hypothesis of intracellular Zn release has gained increased attention (30).

There is compelling evidence that changes in Zn homeostasis are strongly linked to neurodegeneration in AD (81). Early stage clinical trials show that Zn chelating agents significantly decrease deposition of amyloid plaques (26). Zn can bind to both APP and Aβ. When Zn binds to APP at Lys 16 (82), it may alter the ability of α-secretase to cleave APP and as a result decrease the production of soluble APPα and increase the production of Aβ. In addition, Aβ may bind Zn at His-6, His-13, and His-14 enhancing aggregation (83). It was also observed by Bush and colleagues that Zn at concentrations above 300 nM induces the aggregation of human $Aβ_{40}$ (83,84). Zn had no effect on aggregation of rat Aβ (84), probably because of the substitution of His-13 in rat Aβ. Liu et al. suggested His-13 may be a crucial residue in the Zn-induced aggregation of human Aβ (85). In addition, Zn activates kinases which are responsible for phosphorylation of the microtubule associated protein tau by p70 S6 kinase and glycogen synthase kinase 3β. Phosphorylated tau is the main component of NFTs, and therefore Zn may play an important role in changes of tau and subsequent NFTs formation (86).

In contrast, studies by Cardoso et al. showed Zn may have a neuroprotective effect against Aβ in a concentration dependent manner (87). One hypothesis of Aβ toxicity considers the disruption of Na^+/K^+ ATPase by Aβ that leads to elevated Ca^{2+} influx and neurodegeneration. Low concentrations of Zn may reduce the toxicity of Aβ by enhancing Na^+/K^+ ATPase enzyme activity (88). In addition, the conformational changes of Aβ caused by Zn may be protective by preventing oxidizing metals (iron and copper) from binding Aβ and preventing the production of H_2O_2 that can further damage the cell (89).

Zinc levels in Alzheimer's disease brain

Studies of Zn distribution in AD brain have been controversial. Deng and Andrasi measured decreased concentrations of Zn in the hippocampus, inferior parietal lobule and visual

cortices of AD brains with inductively coupled plasma atomic emission spectroscopy (ICP-AES) and instrumental neutron activation analysis (INAA) (27,90). The results were consistent with the study by Panayi and colleagues who reported depletion of Zn in combined brain regions of AD with inductively coupled plasma mass spectroscopy (ICP-MS) (91).

In contrast, Deibel et al. using INAA found Zn significantly elevated in the brain regions which are the most affected in AD: amygdala, hippocampus and inferior parietal lobule (92). Two years later, 58 AD brains were compared to 21 normal brains using INAA and the results demonstrated an increase of Zn in AD (93). Danscher et al. reported elevations of Zn in cryostat sections of hippocampus and amygdala (94). Studies from our laboratory showed an elevation of Zn in senile plaques and neuropil of AD amygdala compared to age matched control neuropil using micro particle-induced X-ray emission (micro-PIXE) (95). Miller and coworkers found Zn accumulation co-localized with Aβ plaques in AD brain using synchrotron X-ray fluorescence microprobe (96). Recent studies by Religa et al. (97) found more than twofold increase of Zn in cortex of AD subjects compared to control subjects. An elevation of Zn levels was accompanied by increased levels of Aβ in the same brain specimens. It is interesting that alterations of Zn concentrations were found in the most vulnerable areas to AD pathology including the hippocampus and amygdala (92–94).

Zinc levels in Alzheimer's disease body fluids

Serum Zn concentrations are approximately 15 μM (58). As with Zn concentrations in the brain, studies of Zn in the serum of AD subjects have been contradictory. A few studies reported no significant difference between AD and control Zn serum levels (98,99). In contrast, Gonzalez et al. found a significant association between higher serum Zn and the presence of the APOε4 allele in AD. This study suggested greater serum Zn concentrations may be an independent risk factor associated with the development of AD (100). Rulon et al. reported a statistically significant elevation of serum Zn in AD subjects compared with age matched control subjects (101). The study by Jeandel et al. showed a decrease of Zn in the serum of AD subjects compared to controls (97). However, other nutrients were also decreased in the serum leading to the speculation that subjects may have been malnourished (102). In addition, a gender sensitive study by Dong et al., in which serum Zn levels from 18 living AD patients, 19 MCI patients and 16 age-matched NC subjects were compared using inductively coupled plasma-mass spectrometry (ICP-MS), showed a significant decrease of serum Zn in men with MCI (103).

Zn levels in cerebrospinal fluid (CSF) are about 0.15 μM (104,105). Studies of Zn levels in CSF of AD patients have been contradictory. Basun et al. showed no significant changes of Zn in the CSF of AD subjects; however, they observed that Zn levels in the blood of AD patients correlated with memory and cognitive functions (106). The recent study by Gerhardsson et al. suggested Zn concentrations in plasma and CSF were not significantly different when comparing AD and control subjects (107). In contrast, Molina et al. reported decreased Zn in AD CSF compared to age matched controls (98).

Potential role of zinc in amyloid hypothesis of Alzheimer's disease

The role of Zn in AD pathogenesis is controversial and is inferred from experimental models (29). The effect of Zn on protein/peptide alterations observed in AD has been extensively studied (108). Pathologically the AD brain is characterized by accumulation of Aβ peptide which results from a proteolytic cleavage of APP. APP mutations observed in familial AD surround the sites of cleavage by three secretases: α-, β-, and γ-secretase. Cleavage by α-secretase leads to the production of soluble APP, whereas cleavage by β-secretase and γ-secretase produces Aβ. Interestingly, Zn can bind to both human APP and Aβ (109). When

Zn binds to APP, it may alter the ability of α-secretase to cleave APP. In addition, cleavage of APP by β- and γ-secretases occurs in intracellular compartments at acidic pH, and Zn ions would make pH more acidic (108). It was also observed by Bush et al. (83,84) that Zn induces the aggregation of Aβ at Zn concentration as low as 0.8 μM. Recent *in vivo* studies showed Zn chelating agents significantly decreased deposition of amyloid plaques in mouse models of Aβ deposition and improved cognition in humans (26, 29,104). Numerous reports demonstrate that Zn can act as a potential mediator of neuronal degeneration (reviewed in 31). Additionally, loss of neurons in epilepsy, ischemia, and traumatic brain injury is characterized by elevations of neuronal Zn (29).

Zinc as potential therapeutic target in Alzheimer's disease

Because Zn plays a potential role in the deposition of Aβ in AD brain, there has been considerable interest in testing of metal-complexing compounds to decrease amyloid pathology in a variety of preclinical systems and clinical trials (110).

Bush and colleagues showed that 9 week long oral treatment with the clioquinol (CQ, 5-chloro-7-iodo-8-hydroxyquinoline) in Tg2576 mice, which overexpress a mutated human APP gene implicated in familial AD, resulted in a reduction of cortical deposition of amyloid (49%) compared to untreated mice (111). CQ can cross the blood–brain barrier (BBB) and was able to increase brain copper and zinc levels in treated mice. In a Phase 2 clinical trial, oral administration of CQ in moderately severe AD patients for 36 weeks slowed the rate of cognitive decline and caused a reduction in plasma $A\beta_{1-42}$ levels as compared to placebo controls (112). CQ clinical trials were terminated due to the contamination of CQ with of a di-iodo derivative during drug manufacture. Additionally, more detailed review of clinical data by Sampson et al. showed CQ did not have any significant effect on cognition (as measured by the ADAS-Cog scale), perhaps because Phase 2 trial were underpowered to detect an effect on cognition (113).

More recently, Prana Biotechnology started clinical trials with a second generation 8-hydroxy quinoline derivative of CQ, PBT-2. During the first double-blind, placebo-controlled Phase 2 clinical trial of 78 AD patients in a 12-week trial for the treatment of mild-moderate AD, AD patients showed improved results for two different cognitive tests and significantly decreased $A\beta_{1-42}$ levels in CSF (113).

Another direction in the development of AD therapeutics is the design of multifunctional molecules that contain both amyloid binding and metal chelating properties (114). Examples include iodine-labeled derivatives of 2-(2-hydroxyphenyl)benzoxazole (HBX), 2-(2-hydroxyphenyl)benzothiazole (HBT), and 2-(2-aminophenyl)-1H-benzimidazole (BM). *In vitro* study by Gonzalez-Duarte et al. showed that these compounds are able to arrest the metal-promoted increase in amyloid fibril buildup (114). Together, these studies suggest changes of Zn concentration may be an effective potential therapeutic target based upon the metal theory of AD.

Conclusions and future directions

Although considerable evidence suggests a link between alterations in Zn and the proteins responsible for its uptake and sequestration in the progression of AD, there is a need for further studies of Zn, ZnTs and other families of Zn binding proteins, ZIPs and MTs, and their relationship to neuropathological hallmarks of AD. Studies of subjects with early stages of AD (PCAD and MCI) are of considerable interest because therapeutic interventions may be more beneficial early in the progression of the disease compared to advanced stages of AD.

To understand the connection between Zn homeostatic proteins and Zn alterations in AD, it might be beneficial to analyze levels of Zn, ZIP proteins and MTs as they relate to ZnT proteins in the AD brain. ZIP proteins are of special interest because they have opposite functions compared to ZnTs and function to transport Zn from the extracellular space or from intracellular vesicles to the cytosol. However, a lack of commercial antibodies for ZIPs coupled with the potential for dispersion of Zn during the post-mortem interval make such studies difficult.

Zinc levels in AD brain were extensively analyzed whereas not many studies quantified extra-parenchymal Zn, especially levels of Zn CSF of subjects with prodromal and early stages of the disease: PCAD, MCI and EAD. Knowing concentrations of Zn in both brain and body fluids and levels of proteins, which regulate Zn balance and correlation between them, would benefit our understanding of alterations of Zn homeostasis in AD.

Another direction for future study is the investigation of the possible relationships between levels of ZnTs and pathological hallmarks of AD, SPs and NFTs. Previous studies suggest Zn and ZnTs may be involved in a complicated mechanism that leads to SPs formation. Lovell et al. reported increased Zn in rims and cores of SPs compared to neuropil concentrations in AD brain (95). Using immunofluorescence staining of human AD brain sections, Zhang et al. found that six ZnTs (1, 3, 4, 5, 6, 7) were extensively present in the Aβ-positive plaques compared to the surrounding tissue in the cortex of human AD brains (115). In contrast, the potential role of Zn in the formation of NFTs was hypothesized and supported by *in vitro* experiments (86) but not addressed *in vivo*.

Our previous studies showed alterations in levels of Zn transporter proteins ZnT-1, ZnT-2, ZnT-4 and ZnT-6 in the brain of subjects with PCAD, MCI, EAD and LAD compared to age-matched NC subjects (57,67,70,71). Because alterations of Zn have been associated with Aβ processing and SPs function we wanted to test whether changes in ZnT proteins and subsequent changes of Zn concentrations alter Aβ processing. To address this hypothesis, we investigated the relationship between protein levels of ZnT-1, ZnT-2, ZnT-4, ZnT-6 and concentrations of Aβ in the media of H4 human neuroglioma cells (H4-APP) transfected to overexpress APP, treated with siRNAs. Our data suggest siRNA mediated attenuation of each induced protein (ZnT-2, ZnT-4 and ZnT-6) leads to decreased production of Aβ (unpublished results). We speculate that because of the function and subcellular localization of ZnTs, they play an important role in the formation of Aβ from APP via cleavage by β- and γ-secretases. Previous studies indicate that Aβ formation is favorable at low pH and occurs in the intracellular space (endosomes or/and lysosomes) (60). By reducing levels of ZnT-2, ZnT-4 and ZnT-6 using siRNAs, the amount of Zn in intracellular organelles, where Aβ processing occurs, is decreased compared to levels in control cells leading to diminished cleavage of APP by β- and γ-secretases and decreased levels of Aβ. Our results support the hypothesis that ZnT mediated sequestration affects the production of Aβ.

There has been considerable interest in the regulation of ZnTs in animal models of AD (116,117). Recent studies by Zhang et al. (117) showed increased ZnT-1, ZnT-4, and ZnT-6 in the hippocampus and neocortex of APPswe/PS1dE9 mice, double transgenic mice overexpressing familial AD-linked APP with Swedish mutation and PS-1 with deletion of exon 9 which demonstrate early Aβ deposition as early as 4–6 months of age that correspond to a form of early onset AD. In addition, ZnT immunostaining was associated with most amyloid plaques in those mice (117). However, there has been limited study of transgenic animal models of AD with genetically altered expression of ZnTs. In the study by Lee et al. (116), ZnT-3 null mice crossed with mice expressing mutant APP led to lower amyloid deposition. To fully elucidate the potential roles of ZnTs in Aβ deposition it would

be beneficial to study ZnT-1, ZnT-2, ZnT-4 and ZnT-6 null mice crossed with AD mutant mice.

Based on reviewed studies of Zn and ZnTs, our working hypothesis is that low systemic Zn early in disease progression may result in elevation of Zn in AD brain leading to alterations of ZnTs and increased concentrations of Zn in subcellular organelles in which Aβ processing occurs; this would cause increased production of Aβ associated with AD. In the course of the disorder resulting alterations in ZnT levels could further contribute to Aβ aggregation and formations of SPs.

Summary

This review discusses the potential role for alterations of zinc (Zn) and Zn transport proteins in the progression of Alzheimer's disease. It describes the normal role of zinc and three major classes of zinc homeostatic proteins (metallothioneins, zinc transporter proteins, and members of zinc-regulated and iron-regulated transporter proteins) in brain along with the potential effects of their alteration on the pathogenesis of AD. The review also addresses studies of zinc toxicity and zinc levels in brain and peripheral body fluids of AD patients, the potential role of Zn in amyloid beta processing and the potential for modulation of Zn as a therapeutic target in AD. Based on reviewed studies, we speculate that low systemic zinc early in disease progression may result in elevated Zn levels in AD brain and subsequent disruption of Zn transporter proteins, leading to increased production and aggregation of amyloid beta peptide.

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