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Alterations in Zinc Transporter Protein-1 (ZnT-1) in the Brain of Subjects with Mild Cognitive Impairment, Early, and Late-Stage Alzheimer's Disease

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Several studies show increased levels of zinc (Zn) in the Alzheimer's disease (AD) brain. More recently, alterations in synaptic Zn and Zn transporter proteins (ZnT) have been implicated in the accumulation of amyloid plaques in an animal model of AD. To determine if alterations in ZnT proteins are present in AD brain, we measured levels of ZnT-1, the protein responsible for export of Zn to the extracellular space in the amygdala (AMY), hippocampus/parahippocampal gyrus (HPG), superior and middle temporal gyrus (SMTG), inferior parietal lobule (IPL), and cerebellum (CER) of 19 AD and 14 age-matched control subjects. To determine if alterations of ZnT-1 occur early in the progression of AD, we analyzed protein levels in the HPG, SMTG and CER of 5 subjects with mild cognitive impairment (MCI), 5 subjects with early AD (EAD) and 4 appropriately age-matched controls. Western blot and dot-blot analysis showed statistically significant (p <0.05) elevations of ZnT-1 in AD AMY, HPG, and IPL and significantly depleted ZnT-1 in AD SMTG compared to age-matched control subjects. We also observed statistically significant elevations of ZnT-1 in the HPG of EAD subjects compared with controls. In contrast to late-stage AD subjects, ZnT-1 levels were significantly decreased in HPG of subjects with MCI and were significantly elevated in the SMTG of both MCI and EAD subjects compared with age-matched controls. Correlation analysis of ZnT-1 levels and senile plaque (SP) and neurofibrillary tangle (NFT) counts in the AMY and CA1 and subiculum of AD HPG showed a significant (p <0.05) positive correlation with SP counts and a trend towards a significant (p = 0.12) positive correlation with NFT counts in AMY. Overall, our results show alterations in one of the key proteins responsible for maintenance of Zn homeostasis early in the progression of AD suggesting that alterations in Zn balance could be involved in the pathogenesis of neuron degeneration and amyloid deposition in AD.

Keywords: Alzheimer's disease; Zinc transporter protein-1; Senile plaques; Neurofibrillary tangles

INTRODUCTION

Alzheimer's disease (AD), a disease of progressive intellectual decline currently affects 4 million Americans (Aging, 1995). Clinically, AD is characterized by a gradually progressive decline in cognitive function and pathologically by neurofibrillary tangle (NFT), amyloid beta peptide (Aß) containing senile plaque (SP) and neuropil thread formation, and neuron and synapse loss, particularly in the hippocampus, amygdala, entorhinal cortex, neocortex and nucleus basalis of Meynert. Of the numerous pathogenic/etiologic mechanisms suggested for the cause of AD, trace element neurotoxicity, particularly zinc (Zn) toxicity has been the focus of considerable interest.

Zinc, an essential element (Golden, 1988; Vallee and Falchuk, 1993), is redox inert with structural, catalytic, and regulatory roles in cellular biology (Bettger and O'Dell, 1981; Golden, 1988; Vallee and Falchuk, 1993) and is a crucial component in over 300 enzymes and transcription factors.

In addition to its essential role, several studies show that elevations of Zn can be toxic to cultured neurons (Gaskin *et al*., 1978; Choi, 1996; Manev *et al*., 1997) and that there are significant elevations of Zn in the AD brain (Danscher *et al*., 1985; 1997; Ehmann *et al*., 1986; Wenstrup *et al*., 1990; Deng *et al*., 1994; Samudralwar *et al*., 1995; Deibel *et al*., 1996).

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Maintenance of Zn homeostasis is achieved through three families of proteins, metallothioneins I - IV (Vallee, 1995) that bind Zn, ZIP (ZRT, IRT-like proteins) that mediate Zn influx into cells (reviewed in Kambe *et al*., 2004) and Zn transporter proteins ZnT-1 through ZnT-7 (reviewed in Kirschke and Huang, 2003) that remove excess intracellular Zn to the extracellular space or sequester Zn in subcellular organelles.

Of the Zn maintenance proteins, zinc transporter proteins are of particular interest. Zinc transporter proteins 1- 7 are members of the cation diffusion facilitator (CDF) family and function to transport Zn out of cells or by sequestering Zn into intracellular compartments (Palmiter and Findley, 1995; Palmiter *et al*., 1996b; Huang and Gitschier, 1997; Cole *et al*., 1999) during periods of elevated Zn. These proteins are predicted to have similar structures including six transmembrane domains and a histidine rich cytoplasmic loop between transmembrane domains IV and V. Of the seven ZnT proteins, ZnT-1, ZnT-3, ZnT-4 and ZnT-6 are expressed in brain.

Because of reported alterations of Zn in AD brain, we analyzed multiple brain areas to determine if alterations in levels of ZnT-1 are present in vulnerable regions of the AD brain. To determine if ZnT-1 alterations occur early in the progression of AD, we measured protein levels in the HPG, SMTG and CER of subjects with mild cognitive impairment and early AD.

MATERIALS AND METHODS

Reagents and Antibodies

Acrylamide and *bis* acrylamide and electrophoresis equipment were purchased from Bio-Rad (Hercules, CA). Nitrocellulose for Western and dot-blots and the dot blot assembly were purchased from Schleicher and Schull (Keene, NH). The ZnT-1 antibody was a rabbit polyclonal generated against a KLH conjugated peptide (GTRPQVSHGKE) representing amino acids 448-458 of the carboxyl terminal of rat ZnT-1 (Chemicon Chemical, Temecula, CA). The sequence used for antibody generation demonstrates a 70% homology with human ZnT-1. Horseradish peroxidase conjugated secondary antibodies were purchased from Amersham Biosciences (Piscataway, NJ). Actin was used as a protein loading control and was visualized using a mouse anti-actin antibody (ABCAM, Cambridge, MA). All other chemicals were from Sigma Chemical (St. Louis, MO).

Brain Specimens

Specimens used for analysis were obtained from the amygdala (AMY), hippocampus/parahippocampal gyrus (HPG), superior and middle temporal gyrus (SMTG), inferior parietal lobule (IPL) and cerebellum (CER) of short *postmortem* interval (PMI) autopsies of 19 AD (5 males, 14 females) and 14 age-matched control subjects (6 males, 8 females). Specimens of HPG, SMTG and CER were also obtained from 5 subjects with mild cognitive impairment (MCI) (2 males, 3 females), 5 subjects with early AD (EAD) (2 males, 3 females) and an additional 4 age-matched control subjects (3 males, 1 female). The brain specimens were immediately frozen in liquid nitrogen and subsequently stored at -80°C until used for analysis. Not all brain regions were available for all subjects. Table I shows subject demographic data. Control subjects were from the longitudinally followed normal control clinic of the University of Kentucky Alzheimer's Disease Center (UK-ADC) and had annual mental status testing, and biannual physical and neurological examinations. AD subjects were from the UK-ADC Research Clinic and had annual mental status testing and physical and neurological examinations. All AD subjects demonstrated progressive intellectual decline and met NINCDS-ADRDA Workgroup (McKhann *et al*., 1984) criteria for the clinical diagnosis of probable AD. Multiple sections of neocortex, hippocampus, entorhinal cortex, amygdala, basal ganglia, nucleus basalis of Meynert, midbrain, pons, medulla, and cerebellum were evaluated for histopathology using hematoxylin and eosin and the modified Bielschowsky stains along with 10D-5 (for Aß) and α-synuclein immunochemistry. All AD patients met accepted criteria for the histopathologic diagnosis of AD (Mirra *et al*., 1991; Aging, 1997), typically demonstrated Braak stage scores of VI, and met the NIA/Reagan Institute high likelihood criteria for the histopathologic diagnosis of AD. Subjects with MCI, and all but one EAD patient, were derived from the control group and were followed longitudinally in the UK-ADC clinic. All MCI patients were normal on enrollement into our longitudinal study and developed MCI during followup. The clinical criteria for diagnosis of amnestic MCI were those of Petersen *et al*. (1999) and included: a) memory complaints, b) expected memory impairment for age and education, c) normal general cognitive function, d) intact activities of daily living (ADLs), and e) the subject did not meet criteria for dementia. Objective memory test impairment was based on a score of ≤ 1.5 standard deviations from the

mean of controls on the CERAD Word List Learning Task (Morris *et al*., 1989) and corroborated in some cases with the Free and Cued Selective Reminding Test. The clinical criteria for EAD were: a) a decline in cognitive function from a previous higher level, b) declines in one or more areas of cognition in addition to memory, c) a clinical dementia rating score of 0.5 to 1 and d) a clinical examination that excluded other causes of dementia. MCI subjects demonstrated Braak staging scores of III to IV. EAD subjects showed Braak staging scores of V and met high likelihood criteria for the histopathologic diagnosis of AD. Histopathologic examination of control subjects as described above revealed only age-associated changes. Control subjects showed Braak staging scores of I to III and met the NIA-Reagan Institute low likelihood criteria for the histopathologic diagnosis of AD. The major difference between normal controls and MCI patients was a significant increase in neuritic plaques in neocortical regions and an increase in neurofibrillary tangles in amygdala, hippocampus and entorhinal cortex (Markesbery *et al*., 2005).

Immunochemistry

To verify specificity of the ZnT-1 antibody, Western blot analyses were carried out on protein samples prepared from a randomly selected specimen of HPG. For Western blot immunochemistry, 100 to 300 mg of tissue was homogenized in 5 ml HEPES buffer (pH 7.4) containing 137 mmol/l NaCl, 4.6 mmol/l KCl, 1.1 mmol/l KH_2PO_4, 0.1 mmol/l $MgSO_4$ and the protease inhibitors leupeptin, pepstatin, aprotinin and PMSF. Tissue was homogenized using a chilled Dounce homogenizer and three ml of the solution centrifuged at 100,000*g* and 4°C for 30 min. The resultant pellet was suspended in buffer and rehomogenized for ZnT-1 measurements. Protein contents were determined using the Pierce BCA method (Sigma, St. Louis, MO).

Protein samples (50 µg) were separated by electrophoresis in 12.5% polyacrylamide gels and transferred to nitrocellulose. The blots were blocked overnight in 5% dry milk and 1.5% goat serum in Tris buffered saline containing 0.5% Triton X-100 (TTBS) and reacted with ZnT-1 antibody at a dilution of 1:500 for 2 h at room temperature. After extensive washing, the blots were incubated with a 1:2000 dilution of horseradish peroxidase conjugated goat anti-rabbit secondary antibody in blocking buffer and bands visualized using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). To test for specificity of the antibodies, representative samples were electrophoresed, transferred to nitrocellulose, and probed with antibody alone or antibody that had been preincubated with immunizing peptide. For preincubation, the antibody was incubated overnight at 37°C with 1 mg immunizing peptide in PBS and centrifuged for 10 min at 15,000*g*. Specimens of HPG, SMTG and CER from MCI, EAD and representative AD and corresponding control subjects were analyzed in the same way. Following analysis for ZnT-1, blots were stripped using Re-blot Plus (Chemicon, Temecula, CA) and re-probed for actin to verify protein loading.

Preparation of Dot-Blots

Based on the specificity of the antibodies and to allow simultaneous comparison of the large number of AD and control subjects, dot-blots for AD/control specimens were prepared by adding 20 µg samples of resolubilized pellet prepared from 19 AD and 14 age-matched control subjects in triplicate using a Schleicher and Schull dot-blot assembly. Following three rinses with PBS, the dot blots were air-dried and treated as described above. The blots were scanned using ADOBE photoshop and staining intensity of each dot measured using SigmaGel software. For statistical analyses, the mean intensity of each dot was calculated and normalized to the mean intensity of control subjects prepared on the same blot. Results are expressed as mean ± SEM percent control staining. Western blots of MCI and EAD specimens and their corresponding controls were scanned using ADOBE photoshop and staining intensity of the bands measured using Scion software (Frederick, MD). For statistical comparisons, intensities of EAD or MCI bands were normalized to the mean control band intensity on each separate gel. Results are expressed as mean ± SEM % control intensity.

Statistical Analysis

Comparison of ZnT-1 staining intensities was carried out using a 2-tailed *t*-test, and the commercially available ABSTAT software. Braak staging scores were compared using non-parametric testing and the Mann-Whitney U-test.

RESULTS

Mean ± SEM age, PMI, SP and NFT counts for AD and age-matched control subjects and age and PMI data for MCI and EAD subjects are shown in Table I. Median Braak staging scores are also shown in Table I. There were no significant differences between MCI, EAD or AD and control subjects in age or PMI. Median Braak staging scores for control subjects were significantly lower (II) compared with MCI (III), EAD (V) and AD

subjects (VI) (p <0.05). Senile plaque and NFT counts were significantly (p <0.03) higher in AD AMY and CA1 and subiculum of HPG compared to age-matched control subjects.

Specificity of the ZnT-1 antibody was evidenced by the appearance of a strong band at 52 kDa consistent with full length ZnT-1 (FIG. 1). We also observed a ZnT-1-positive band at a slightly higher molecular weight that may be due to post-translational modification of ZnT-1. Preincubation of the ZnT-1 antibody with immunizing peptide showed elimination of antibody staining (FIGs. 1B). Figure 2 shows representative Western blots from AD and control HPG probed for ZnT-1 and ß-actin as a loading control.

Figure 3 shows a statistically significant (p <0.05) increase in ZnT-1 protein levels in AD AMY (183.5 ± 23.5% control; n=9) compared with control AMY (100 ± 25.6%; n=7), AD HPG (185.4 ± 27.6%; n=14) compared with control HPG (100 ± 7.0%; n=13), and AD IPL (157.4 ± 21.6; n=16) compared with control IPL (100 ± 13.1%; n=13). In contrast, ZnT-1 levels were significantly depleted in AD SMTG (73.5 ± 8.3%; n=16) compared with control SMTG (100 ± 7.2% n=13). There was no significant difference in ZnT-1 levels in CER between AD and control subjects. Table II shows levels of ZnT-1 were also significantly elevated in EAD HPG (135.4 ± 3.3% control) compared to age-matched controls (100 ± 3.1%; p <0.01) and in EAD SMTG (133.5 ± 3.3% control) compared to control subjects (100 ± 2.4%; p <0.01). Specimens of MCI SMTG also showed significantly higher ZnT-1 levels (140.5 ± 5.5% control) compared with control SMTG (100 ± 2.9%; p <0.01). In contrast, MCI HPG showed a significant decrease in levels of ZnT-1 (31.8 ± 10.6% control) compared to control HPG (100 ± 3.1 %; p <0.02).

Correlation analyses of ZnT-1 levels and NFT and SP counts in AMY and CA1 and subiculum of HPG of AD and control subjects showed a significant (p = 0.01) positive relationship between ZnT-1 and SP counts in the AMY. No other correlations were significant.

DISCUSSION

Of the essential trace elements in the human body, Zn is of particular interest. It is redox inert and serves structural, catalytic and regulatory roles in cell biology. In the brain, Zn occurs in three distinct pools including a membrane bound metalloprotein pool, an ionic pool of loosely bound ions in the cytoplasm, and a vesicular pool released during neurotransmission (Haug, 1967; Frederickson *et al*., 1983; Perez-Clausell and Danscher,

Table I

	Age (years)	PMI (hours)	Braak Score	CA-1		Subiculum		Amygdala	
				SP	NFT	SP	NFT	SP	NFT
MCI	88.8 ± 2.0	3.0 ± 0.5	III*	—	—	—	—	—	—
EAD	89.2 ± 2.1	2.9 ± 0.4	V*	—	—	—	—	—	—
AD	81.4 ± 2.1	2.8 ± 0.1	VI*	3.6 ±0.9*	32.9 ± 5.1*	8.3 ± 2.3*	40.8 ± 4.4*	24.5 ± 2.7*	43.5 ± 5.3*
Control	8.6 ± 3.9	3.0 ± 0.2	II*	1.0 ± 0.5	6.3 ± 1.8	2.0 ± 1.1	10.0 ± 5.5	7.9 ± 2.6	10.0 ± 5.5

*p <0.05

Table II

	MCI ZnT-1 (% Control)	Control ZnT-1 (% Control)	EAD ZnT-1 (% Control)	Control ZnT-1 (% Control)
HPG	31.8 ± 10.6%*	100 ± 3.1%	135.4 ± 3.3%*	100 ± 3.1%
SMTG	140.5 ± 5.5%*	100 ± 2.9%	133.5 ±4.2%*	100 ± 2.4%
CER	102.5 ± 1.9%	100 ± 11.7%	92.5 ± 6.5%	100 ± 2.9%

*p <0.02

1985; 1986; Cuajungco and Lees, 1997). Gray matter Zn concentrations range between 50 and 200 µM (Ehmann *et al*., 1986) with concentration gradients ranging from < 10^{-9} M in the neuron cytoplasm to > 10^{-3} M in synaptic vesicles (Williams, 1989). During neurotransmission, Zn is released into the extracellular space and may reach concentrations in excess of 100 µM (Assaf and Chung, 1984; Cole *et al*., 1999) which are toxic to primary neuron cultures (Gaskin *et al*., 1978; Choi, 1996; Manev *et al*., 1997).

Although Zn is essential for normal function of the brain, several studies show significant elevations of Zn in the AD brain, both at the bulk level (Danscher *et al*., 1985; 1997; Ehmann *et al*., 1986; Wenstrup *et al*., 1990; Deng *et al*., 1994; Samudralwar *et al*., 1995; Deibel *et al*., 1996) and in senile plaques (Lovell *et al*., 1998). Additionally, the vesicular Zn pool is most abundant in the olfactory bulb, cerebral cortex, and limbic areas of the brain and is relatively low in the cerebellum (Perez-Clausell, 1996; Danscher *et al*., 1997; Frederickson *et al*., 2000; Jo *et al*., 2000); a regional distribution pattern that correlates well with the degree of AD pathology. Zn may also contribute to the pathogenesis of AD through its ability to mediate aggregation of the Aß (Bush *et al*., 1994; Huang *et al*., 1997).

Because the brain is subject to wide concentration gradients of Zn that can be neurotoxic, it is imperative that cells regulate Zn levels through control of influx and efflux and through chelation to sequestering proteins (Palmiter *et al*., 1992; 1996a,b; Ebadi *et al*., 1995; Palmiter, 1995; Palmiter and Findley, 1995; Cuajungco and Lees, 1997). In general, Zn homeostasis is maintained through three families of Zn binding proteins, metallothioneins (MT) I - IV, ZIP proteins (Kambe *et al*., 2004), and Zn-transporters ZnT-1 through ZnT-7. Although there have been several studies of the role of MTs in AD, there have been no studies of ZnT proteins.

Our study shows there are statistically significant elevations of ZnT-1 protein in AD AMY, HPG and IPL but a significant decrease in AD SMTG compared with age-matched control subjects. Comparison of ZnT-1 levels and SP and NFT counts in the AMY of AD subjects shows a positive relationship between ZnT-1 protein levels and SP and NFT counts in the AMY. Our data also show a statistically significant decrease in ZnT-1 in AD SMTG. We also observed statistically significant elevations of ZnT-1 in the HPG of EAD subjects. In contrast to late-stage AD, we observed statistically significant elevations of ZnT-1 in the SMTG of MCI and EAD subjects. In a somewhat surprising finding, we observed a significant decrease in ZnT-1

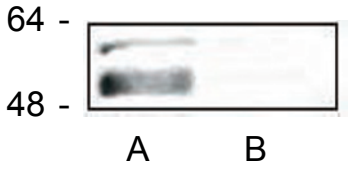

FIGURE 1A The ZnT-1 antibody was specific for full length ZnT-1 at 52 kDa.
FIGURE 1B. Staining of the ZnT-1 band was significantly decreased by preincubation of the antibody with immunizing peptide.

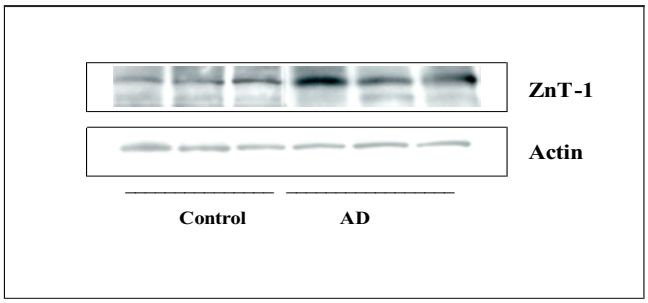

FIGURE 2. Representative Western blots of HPG from AD and age-matched control subjects probed for ZnT-1 and actin as a loading control.

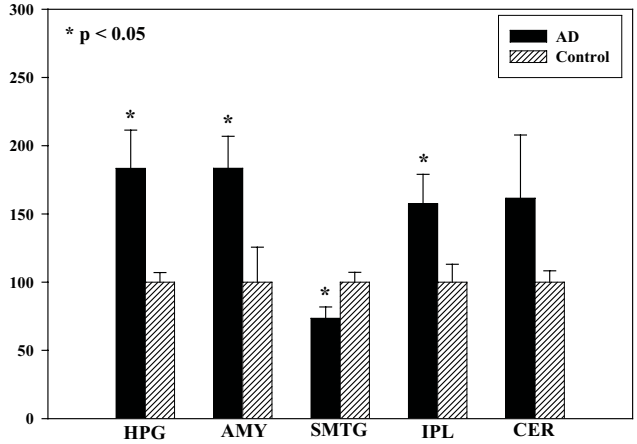

FIGURE 3. ZnT-1 protein levels expressed as mean ± SEM % control. There was a significant (p <0.05; 2 tailed t-test) elevation of ZnT-1 in AD AMY, HPG and IPLC and a significant decrease in AD SMTG compared to age matched control subjects.

in MCI HPG.

ZnT-1 is located in the plasma membrane and functions to export Zn from the cell (Palmiter and Findley, 1995) during periods of Zn release from synaptic vesicles and confers resistance to Zn in transfected cells. Although ZnT-1 was the first ZnT protein characterized (Palmiter and Findley, 1995), there have been no studies of its expression in human brain, particularly in AD brain.

Our data show a statistically significant decrease of ZnT-1 in MCI HPG but statistically significant elevations in early and late-stage AD. Although speculative, the observation of diminished ZnT-1 in MCI, the earliest clinical manifestation of AD, suggests that the maintenance of Zn balance is altered early in AD and may play a role in the pathogenesis of neuron degeneration. It is possible that the decrease in ZnT-1 levels in MCI may allow a buildup of intracellular Zn levels. Because other Zn transporters, particularly ZnT-3, 4 and 6 can sequester intracellular Zn in the brain, it is possible that the initial loss of ZnT-1 mediated Zn efflux is not detrimental to the cells. However, after prolonged intracellular Zn, the cells may no longer be able to tolerate elevated Zn levels and may increase ZnT-1 synthesis. This increased ZnT-1 expression and subsequent efflux of Zn could result in elevations in the extracellular space that could interact with Aß leading to increased precipitation and senile plaque deposition. Indeed, several studies show that Zn plays a role in the reversible precipitation of Aß (Bush *et al*., 1994; Huang *et al*., 1997) and can directly bind Aß in AD senile plaques through histidine bridges (Dong *et al*., 2003). Our observation of a positive correlation between ZnT-1 and SP and NFT in AMY support the potential contribution of altered Zn homeostasis and SP formation and neuron degeneration.

Overall, our data show significant alterations in levels of one of the key zinc transport proteins in vulnerable regions of MCI, EAD and AD brain. Our data coupled with observations of elevated Zn in vulnerable regions of AD brain suggest that alterations in proteins that maintain Zn homeostasis may contribute to increased neuron degeneration through elevated Zn levels in the synaptic cleft. Additionally, increased extracellular Zn could play a role in the precipitation of Aß and increased senile plaque deposition.

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