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ALTERATIONS OF ZINC TRANSPORTERS IN ALZHEIMER'S DISEASE

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ABSTRACT OF DISSERTATION

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The Graduate School
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A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences
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By

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Lexington, Kentucky

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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. We hypothesize that disruption of Zn homeostasis leads to alterations of Zn transporter (ZnT) proteins, resulting in increased production of neurotoxic amyloid beta (Aβ) peptide in AD brain. To address this hypothesis we carried out the following studies.

1. We characterized alterations of ZnT-1, ZnT-4 and ZnT-6 in the brain of preclinical AD (PCAD) subjects, who show no overt clinical manifestations of AD but demonstrate significant AD pathology at autopsy.
2. We identified the presence of ZnT-2 in human brain and compared protein levels in the brains of subjects with PCAD, mild cognitive impairment (MCI), early (EAD), and late-stage AD (LAD) to those in age matched normal control (NC) subjects.
3. We examined the relationship between protein levels of ZnT-1, ZnT-2, ZnT-4, ZnT-6 and Aβ produced by H4 human neuroglioma cells (H4-APP) transfected to overexpress amyloid precursor protein (APP), treated with short interfering RNA (siRNA) against each ZnT.

Our data show a significant decrease ($P < 0.05$) of ZnT-1 and a significant increase of ZnT-6 in hippocampus/parahippo-campal gyrus (HPG) of PCAD subjects. In PCAD cerebellum (CER) the data show a significant increase of ZnT-4 and ZnT-6 compared to NC subjects. Levels of ZnT-2 were also significantly decreased in HPG of PCAD subjects compared to NC subjects. In addition, levels of ZnT-2 were significantly ($P < 0.05$) elevated in SMTG of PCAD and MCI subjects, compared to NC subjects. ZnT-2 was significantly ($P < 0.05$) elevated in HPG of EAD and LAD, and in SMTG of LAD brains, but was significantly ($P < 0.05$) decreased in LAD CER compared to NC subjects. siRNA mediated attenuation of each ZnT protein studied (ZnT-2, ZnT-4 and ZnT-6) led to significantly ($P < 0.05$) decreased production of Aβ compared to controls.

Our results suggest alterations in Zn transport may play a role in Aβ processing and contribute to the neuropathology of AD.

KEYWORDS: Alzheimer's disease (AD), amyloid beta (Aβ), preclinical Alzheimer's disease (PCAD), zinc (Zn), zinc transporter (ZnT).

Ganna Lyubartseva

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CHAPTER ONE

Introduction and background

1.1 Alzheimer's disease (AD)

1.1.1 Discovery and incidence

In 1901, German psychiatrist Dr. Alois Alzheimer diagnosed a patient (51- year-old Mrs. Auguste Deter) with "amnesic writing disorder" a disease that was later named for Dr. Alzheimer (Hodges, 2006). The patient suffered from severely impaired memory, aphasia, erratic behavior, paranoia and auditory hallucinations and died demented in 1906. Autopsy of the brain showed neuronal loss along with intracellular aggregates of the protein later identified as hyperphosphorylated tau (tangles) as well as extracellular deposits of protein later identified as β-amyloid (plaques). These two lesions remain the main pathological hallmark features of Alzheimer's disease (AD).

Currently about 6% of the population over age 65 suffers from AD and incidence rates increase with age (Burns and Iliffe, 2009). At the beginning of the twenty-first century, AD is the most common form of adult-onset dementia, and currently affects 4.5 million Americans (Hebert et al., 2003) and may affect 13 million by 2050, unless preventive strategies are found. AD is the eighth leading cause of death in the United States (Hoyert et al., 2006) with direct and indirect medical and social costs in excess of $100 billion per year (Yaari and Corey-Bloom, 2007).

1.1.2 Clinical characteristics

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 (ADLs) (Burns and Iliffe, 2009). The first group (cognitive dysfunction) includes memory loss, language difficulties, as well as executive dysfunction characterized by a loss of higher level planning and intellectual coordination skills. Non-cognitive symptoms include psychiatric and behavioral disturbances, often manifested as depression, hallucinations and agitation. The third group includes difficulties in performance of ADLs as well as more complex activities such as driving and shopping.

The symptoms of AD progress from mild symptoms of memory loss to profound dementia. In general, AD patients die from secondary infections and illnesses (Burns and Iliffe, 2009). Data from the Baltimore Longitudinal Study of Aging suggest median survival time following diagnosis of AD depends on the patient's age at diagnosis and ranges from 8.3 years for persons diagnosed as having AD at age 65 years to 3.4 years for persons diagnosed as having AD at age 90 (Brookmeyer et al., 2002).

Clinical diagnostic criteria for the AD include those defined by the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) and National Institute of Neurological Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) (Yaari and Corey-Bloom, 2007). In addition, clinical dementia rating (CDR) scores are often used as a clinical tool to differentiate healthy aging from clinical dementing illness (Price, 2003). CDR scores range from 0 (cognitively healthy) to 3.0 (profound dementia), with specific intervals used to identify different stages of AD (Table 1.1). According to DSM-IV, AD is characterized by an insidious onset with continuing decline of cognitive function that results in impairment of social and occupational function, memory impairment and at least one other cognitive deficit. The NINCDS-ADRDA criteria are more detailed and allow diagnosis of AD with different levels of likelihood (McKhann et al., 1984). The diagnosis of "probable" AD is based on the following main criteria:

- Dementia established by clinical examination and confirmed by neuropsychological testing;
- Deficits in two or more areas of cognitive function;
- Progressive worsening of memory or other cognitive functions;
- No disturbance of consciousness;
- Onset between ages 40 and 90;
- Absence of systemic disorders or other brain diseases that could account for progressive memory and cognitive changes;

Other criteria include the following:

- Impaired ADLs and altered behavior;
- Family history of similar disorder;
- Plateaus in the course of the disease;

- Associated symptoms of depression, insomnia, etc.

However, these are criteria for the "probable" diagnosis of AD only. The major challenge associated with AD is that definite diagnosis is only possible at autopsy (Yaari and Corey-Bloom, 2007). Neuropsychological tests such as volumetric measurements of the hippocampus and related structures using magnetic resonance imaging (MRI), tomography, electrophysiological procedures, and the study of body fluids and nonneural tissues are currently used to diagnose AD on the basis of exclusion (McKhann et al., 1984).

1.1.3 Pathological features

Gross examination of AD brain typically shows marked atrophy, with widened sulci and shrinkage of the gyri (Yaari and Corey-Bloom, 2007). Neuropathologically, the AD brain demonstrates selective neuronal and synapse loss, particularly in the hippocampus, amygdala, entorhinal cortex, neocortex and nucleus basalis of Meynert, and an abundance of senile plaques (SP) composed of amyloid beta peptide (Aβ), a product of amyloid precursor peptide (APP) proteolytic cleavage (Figure 1.1), and neurofibrillary tangles (NFT) containing hyperphosphorylated tau (Figure 1.2). 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).

Postmortem histopathological diagnosis of AD is made using National Institute on Aging–National Institute of Neurological and Communicative Disorders and Stroke (NIA-NINCDS) or Khachaturian criteria, Consortium to Establish a Registry for Alzheimer's Disease (CERAD) and NIA-Reagan criteria (Yaari and Corey-Bloom, 2007). NIA-NINCDS criteria require certain levels of neocortical plaque density adjusted for age to diagnose AD, although they do not specify plaque type or neocortical region. The CERAD criteria include quantification of neuritic plaques in different neocortical regions and classification of plaque densities into sparse, moderate or frequent categories. These criteria work in the context of three age groups: less than 50, 50-75 and older than 75. NIA-Reagan criteria combine CERAD criteria and the quantification of NFTs using

Braak staging (Anonymous, 1997). Braak scores evaluate NFT density and distribution as they spread from the transentorhinal cortex, to the hippocampus and finally to the neocortex; and permit differentiation of six stages (I to VI) where the higher stage is associated with the higher degree of NFT pathology. NIA-Reagan criteria for the diagnosis of probable AD require frequent plaques according to CERAD criteria and NFTs in neocortex (Braak V-VI). For the diagnosis of probable AD, the brain should demonstrate moderate neuritic plaques and NFTs in the hippocampal gyri. CERAD and NIA-Reagan criteria together with clinical information allow diagnosis of definite, probable and possible AD.

1.1.4 Risk factors

Although the cause of AD is unknown, numerous studies have linked several risk factors with the disease (Yaari and Corey-Bloom, 2007; Burns and Iliffe, 2009). Age is considered to be the main risk factor for AD (Yaari and Corey-Bloom, 2007). Both prevalence and incidence of AD rise dramatically after age 65, and the prevalence doubles approximately every 5 years between 65 and 95. Prevalence increases from ~2% in those aged 65 to 69, to 4% in those aged 70 to 74, to 8% in those aged 75 to 79, to 16% in those aged 80 to 85, and to ~35% to 40% in those over the age of 85 (Yaari and Corey-Bloom, 2007).

The second most important risk factor for AD is a positive family history (Yaari and Corey-Bloom, 2007). The risk for first degree relatives of people with the disease is 10-40% higher than in unrelated people (Burns and Iliffe, 2009). Twin studies have found that AD precedence is higher in monozygotic twins than in dizygotic twins, suggesting the presence of a genetic component (Raiha et al., 1997).

However, the modest concordance levels in monozygotic twins who share 100% genetic material suggests an existence of environmental factors for AD (Burns and Iliffe, 2009). Other possible risk factors for AD include gender (Zhang et al., 1990), low levels of education (Zhang et al., 1990; Riley et al., 2005), low linguistic ability in early life (Zhang et al., 1990; Snowdon et al., 2000; Riley et al., 2005), head injury (Mortimer et al., 1991; Fleminger, 2008), hypertension (Iadecola et al., 2009), diabetes (Irie et al., 2008) and depression (Leontjevas et al., 2009).

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, and amyloid precursor (APP) on chromosome 21 (Xie and Tanzi, 2006). Most people with Down's syndrome develop the neuropathological features of AD by the age of 40, probably due to having an extra copy of the APP gene (Mann et al., 1990; Cataldo et al., 2004).

Apolipoprotein E (ApoE) is a susceptibility factor for AD (Corder et al., 1993). ApoE is involved in cholesterol transport and probably neuronal repair, and exists as three alleles: ε2, ε 3, and ε4 (Yaari and Corey-Bloom, 2007). 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. Among Caucasians, the ε4/ ε4 genotype has been associated with a fifteen times higher risk of AD compared with the ε3/ ε3 genotype (Yaari and Corey-Bloom, 2007). The ε4/ ε3 genotype has been associated with three times higher risk (Yaari and Corey-Bloom, 2007). However, the mechanism by which an individual's ApoE gene status affects the risk of developing AD is unknown (Corder et al., 1993).

1.1.5 Early stages

There is a considerable interest in understanding how AD progresses and 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 (Table 1.1) (Yaari and Corey-Bloom, 2007).

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 (Lange et al., 2002; Fagan et al., 2005). 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 (Braak and Braak, 1997). Another study showed amyloid plaques in the neocortex distinguish early stages of AD from normal brain aging (Morris and Price, 2001).

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 (Flicker et al., 1991). 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 (Petersen, 2007). Petersen's criteria for clinical diagnosis of amnesic MCI include:

1. Memory complaints corroborated by an informant
2. Abnormal memory impairment adjusted for age and education
3. Normal general cognitive function
4. Intact ADLs
5. The subject does not meet criteria for dementia.

Longitudinal studies suggest that cognitive impairments in this early stage may remain relatively constant for several years (Petersen, 2007). The stable phase of MCI ends with a detectable decline in cognitive function, lasting two to five years (Spaan et al., 2003). Prospective studies have shown that people with amnesic MCI are fifteen times more likely to have developed dementia at follow-up, suggesting it may be a precursor to AD (Burns and Iliffe, 2009).

In addition to the concept of amnesic MCI as an early stage of AD with a high rate of progression to 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 (Knopman et al., 2003; Fagan et al., 2005; Galvin et al., 2005). Clinical and histopathological criteria for four stages of AD (PCAD, MCI, EAD and LAD) are described in Table 1.1.

1.1.6 Etiology

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 (Markesbery, 1997), the amyloid hypothesis (reviewed in Marcello et al., 2008), and the toxic metal hypothesis (Cuajungco and Faget, 2003) among others. However, none of the individual

hypotheses satisfactorily explain the clinical and pathological picture of AD. A combination of theories should be considered in order to explain the origin and manifestation of the disease.

In recent years the toxic metal hypothesis of AD has received renewed interest. Among most studied metals are aluminum (Al), iron (Fe), copper (Cu), and zinc (Zn) (discussed in later section).

The Al hypothesis of AD was initiated by Klatzo et al. (Klatzo et al., 1965) who observed formation of apparent NFTs following intracerebral injection of Al salts into rabbit brains. Similar results in cats were reported by Crapper et al. (Crapper et al., 1973). Initial bulk studies of AD brain using atomic absorption spectroscopy (AAS) showed increased Al compared to controls (Boni et al., 1976; Trapp et al., 1978). The hypothesis of elevated Al in AD brain was supported by data from instrumental neutron activation analysis (INAA) studies (Yasui et al., 1980; Parkinson et al., 1981). In contrast, AAS measurements (McDermott et al., 1979), INAA bulk studies (Markesbery et al., 1981), energy dispersive X-ray microanalysis (EDX) (Jacobs et al., 1989) and studies of neurofibrillary tangle (NFT)-bearing neurons by Lovell et al. using microprobe analysis (Lovell et al., 1993) showed no differences in Al brain levels between AD and controls. The role of Al in the pathology of AD was disputed in later reports because NFTs in animal models were made of straight filaments, but in AD are composed of paired helical filaments (Munoz-Garcia et al., 1986) and because there was no obvious correlation between the degree of neurofibrillary changes and the severity of the clinical signs and symptoms (Wisniewski et al., 1980).

The hypothesis that Fe may be involved in the pathogenesis of AD was based on the speculation that elevations of Fe could accelerate oxidative damage and promote neurodegeneration; although, AD clinical trials using Fe chelating agents such as desferroxamine have met with limited success (Castellani et al., 2007).

Another trace metal with both positive and negative reports of imbalance in AD is Cu. Independent studies by Deibel et al. (Deibel et al., 1996) and Loeffler et al. (Loeffler et al., 1996) showed decreased Cu levels in AD brain compared to controls. In contrast, a micro particle-induced X-ray emission (micro-PIXE) study by Lovell et al. found Cu significantly elevated in the rim of SPs compared with AD neuropil (Lovell et al., 1998).

Cu is a part of the active site of the antioxidant enzyme Cu/Zn superoxide dismutase (reviewed in Macreadie, 2008). Studies by Omar et al. suggest increased expression but reduced activity of Cu/Zn superoxide dismutase in AD brain (Omar et al., 1999). Bayer et al. showed chronic amyloid precursor (APP) overexpression *per se* reduced Cu/Zn superoxide dismutase activity in transgenic mouse brain, however the activity could be restored to normal levels after Cu supplementation, suggesting decreased activity of the enzyme may be due to depleted Cu levels in AD brains (Bayer et al., 2003).

To summarize, reports on alterations of trace metals in AD have been controversial probably because various analytical methods were employed, different types of analyses were conducted (bulk vs. microprobe), and numbers of subjects were not comparable across different studies.

1.2 The role of zinc (Zn) in AD

1.2.1 Metabolic role of Zn

Trace elements play an important role in metabolism (Andrasi et al., 1995). The presence of nine trace elements: iron, Zn, copper, selenium, iodide, manganese, molybdenum, chromium and cobalt are considered as the most critical for human health (Hambidge, 2003). A role for trace metals has been proposed for the pathogenesis of neurological disorders such as Wilson's disease and Parkinson's disease (Frederickson et al., 1983). Recent studies suggest disruptions of trace elements are involved in the neuropathology of AD (Cuajungco and Faget, 2003; Capasso et al., 2005; Frederickson et al., 2005).

In the past few decades, a variety of structural, catalytic and regulatory functions of Zn have been described and Zn is often called “the calcium of the twenty-first century” (Frederickson et al., 2005). Zn is the second most abundant trace element (concentration of µg/g of wet tissue) in the body after iron (Frazzini et al., 2006). Crucial decisions about cellular growth, proliferation, differentiation and programmed cell death involve Zn in ionic or protein-bound forms (Cuajungco and Faget, 2003). Zn moderates the activity of at least 300 enzymes and transcription factors and plays an important role in DNA and RNA transcription and replication (Vallee and Falchuk, 1993). Zn is also

involved at all levels of signal transduction in mammalian cells (Beyersmann and Haase, 2001).

A majority of total body Zn (85%) is in muscle and bone, 11% in skin and the liver and the remaining in all the other tissues (Stefanidou et al., 2006). The average Zn content of the adult human body is about 1.4–2.3 g (Stefanidou et al., 2006). The recommended daily allowance of Zn for adults is 15 mg and 3–5 mg for infants (Barceloux, 1999). Ingested Zn is absorbed by the intestines and is eliminated primarily via pancreatic and liver excretion (70–80%), with relatively little Zn appearing in the urine or sweat (15–25%) (Barceloux, 1999). Radioactive Zn labeling studies show ~ 1% orally administered Zn is eliminated daily (Sullivan and Heaney, 1970). Zn levels in the body are tightly regulated and excretion of Zn increases as the intake increases (Barceloux, 1999).

Zn is an essential element and its deficiency leads to a wide range of symptoms including effects in the central nervous system (CNS) (Zatta et al., 2003). Symptoms of Zn deficiency include distorted or absent taste, and disruptions of smell and vision. Deficiencies in Zn accompany diseases such as gastrointestinal disorders, renal disease, sickle cell anaemia, alcoholism, some cancer types, acquired immunodeficiency syndrome (AIDS) and Down's syndrome (Stefanidou et al., 2006). An abnormally low Zn concentration in breast milk has been associated with the development of Zn deficiency in breast-fed newborns characterized by acrodermatitis, irritability, and delayed growth (Barceloux, 1999). Prolonged Zn deficiency has been associated with hypogonadal dwarf syndrome which is characterized by growth retardation, delayed sexual maturation, iron-deficiency anemia, poor wound healing, alterations of taste and simultaneous enlargement of both the liver and the spleen (Barceloux, 1999). Data from National Health and Nutrition Examination Surveys in the United States indicate that adults over age 70 represent a population group at greatest risk of inadequate intake of Zn (Briefel and Johnson, 2004). Results of European ZINCAGE project showed that the elderly population in general has insufficient Zn uptake suggesting a relationship between plasma Zn levels and cognitive decline (Marcellini et al., 2006).

There are two inherited disorders which are associated with alterations in Zn homeostasis. The first, a dermatological disorder of Zn deficiency, acrodermatitis

enteropathica, is manifested by hair loss, ulcerated skin, chronic diarrhea, and muscle wasting (Stefanidou et al., 2006). The second disorder is familial hyperzincaemia, in which Zn plasma levels are abnormally high. Hyperzincaemia has no specific associated symptoms; therefore, its prevalence is unclear (Selimoglu et al., 2006).

Toxicity associated with excessive exposure to Zn is less well documented (reviewed by Zatta et al., 2003). Situations in which toxicity has been observed include inhalation of Zn fumes, deliberate ingestion, and exposure to contaminated food and/or drinking water. Involvement of the nervous system in some cases of Zn toxicity in humans has also been described. Lethargy, light-headedness and loss of neuromuscular coordination are symptoms of Zn intoxication and are usually eliminated upon removal of the excess Zn (Zatta et al., 2003).

1.2.2 Zn in mammalian brain

The mammalian brain contains ~10-20 μM Zn (Frederickson, 1989). Zn concentrations in gray matter vary from 150 to 200 μM (Ehmann et al., 1986). The concentration of free Zn ions in the extracellular space of healthy brain tissue is in the range of 1 to 10 nM (Frederickson et al., 2005), 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 (Assaf and Chung, 1984) to 300 μM in synaptic cleft (Maynard et al., 2005).

In neurons, Zn stabilizes glutamate in synaptic vesicles and modulates the behavior of postsynaptic membrane receptors and ion channels (Zatta et al., 2003). 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 (Frederickson et al., 2005). The study by Takeda et al. demonstrated that rats on Zn deficient diet for four weeks did not have significant changes in Zn levels in hippocampus compared to controls (Takeda et al., 2005), 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 (Chowanadisai et al., 2005). It is believed, that even though Zn is necessary for normal cellular function, excessive intracellular Zn concentrations lead to inactivation of vital cellular processes. Studies by Ducray et al. showed excessive Zn concentrations are toxic for neurons. $ZnSO_4$ administered by intranasal perfusion to mice led to total destruction of the olfactory neurons in a few days (Ducray et al., 2002). However, tissue and function restoration occurred in the following weeks.

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 (Frederickson, 1989). Chelatable Zn is present in the hippocampus, amygdala, visual, and somatosensory cortex (Frazzini et al., 2006).

1.2.3 Zn levels in AD brain

Studies of Zn distributions in AD brains 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) (Deng et al., 1994; Andrasi et al., 1995). 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) (Panayi et al., 2002).

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 (Deibel et al., 1996). Two years later, 58 AD brains were compared to 21 normal brains using INAA and the results demonstrated an increase of Zn in AD (Cornett et al., 1998). Danscher et al. reported elevations of Zn in cryostat sections of hippocampus and amygdala (Danscher et al., 1997). 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) (Lovell et al., 1998). Miller and coworkers found Zn accumulation co-localized with Aβ plaques in AD brain using synchrotron X-ray fluorescence microprobe (Miller et al., 2006). Recent studies by Religa et al. (Religa et al., 2006) 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 (Deibel et al., 1996; Danscher et al., 1997; Cornett et al., 1998).

1.2.4 Zn levels in AD body fluids

Serum Zn concentrations are approximately 15 μM (Takeda, 2000). 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 (Shore et al., 1984; Molina et al., 1998). 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 (Gonzalez et al., 1999). Rulon et al. reported a statistically significant elevation of serum Zn in AD subjects compared with age matched control subjects (Rulon et al., 2000). The study by Jeandel et al. showed a decrease of Zn in the serum of AD subjects compared to controls (1989). However, other nutrients were also decreased in the serum leading to the speculation that subjects may have been malnourished (Jeandel et al., 1989). 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 normal control subjects were compared using inductively coupled plasma-mass spectrometry (ICP-MS), showed a significant decrease of serum Zn in men with MCI (Dong et al., 2008).

Zn levels in cerebrospinal fluid (CSF) are about 0.15 μM (Hershey et al., 1983; Palm et al., 1986). 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 (Basun et al., 1991). The recent study by Gerhardsson et al. suggested Zn concentrations in plasma and CSF were not significantly different when comparing AD and control subjects (Gerhardsson et al., 2008). In contrast, Molina et al. reported decreased Zn in AD CSF compared to age matched controls (Molina et al., 1998).

1.2.5 Toxicity of Zn in AD

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 (Yokoyama et al., 1986; Ducray et al., 2002). Cell culture studies by Yokoyama et al. showed 15 min exposure to 300–600 μM Zn caused extensive cortical neuron death (Yokoyama et al., 1986). 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 (Ducray et al., 2002).

Although mechanisms of Zn mediated cell death are unclear, numerous hypothetical mechanisms have been proposed (Zatta et al., 2003; Capasso et al., 2005). The dominant theory of Zn accumulation and neurotoxicity has been the hypothesis of Zn translocation (Zatta et al., 2003; Capasso et al., 2005). 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 (Frederickson et al., 2005). 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 (Sensi et al., 1997). Other studies found that Zn^{2+} may act as a neurotransmitter and mediate apoptosis (Capasso et al., 2005). Recently, the hypothesis of intracellular Zn release has gained increased attention (Capasso et al., 2005).

There is compelling evidence that changes in Zn homeostasis are strongly linked to neurodegeneration in AD (Markesbery and Ehmann, 1994; Bush et al., 1995; Lovell et al., 1998; Cuajungco and Faget, 2003; Lovell et al., 2005; Lovell et al., 2006; Frazzini et al., 2006). Clinical trials show that Zn chelating agents significantly decrease deposition of amyloid plaques (Cuajungco and Faget, 2003). Zn can bind to both amyloid precursor protein (APP) and Aβ. When Zn binds to APP at Lys 16 (Esch et al., 1990), 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 can bind Zn at His-6, His-13, and His-14 (Figure 1.3) (Bush et al., 1993). It was also observed by Bush and colleagues that Zn at concentrations above 300 nM induces the aggregation of human $A\beta_{40}$ (Bush et al., 1993; Bush et al., 1994b). Zn had no effect on aggregation of rat Aβ (Bush et al.,

1994b), probably because of the substitution of His-13 in rat Aβ (Figure 1.3). Liu et al. suggested His-13 may be a crucial residue in the Zn-induced aggregation of human Aβ (Liu et al., 1999). In addition, Zn activates kinases which are responsible for phosphorylation of tau protein 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 (An et al., 2005).

In contrast, the work by Cardoso et al. showed Zn may have a neuroprotective effect against Aβ in a concentration dependent manner (Cardoso et al., 2005). One hypothesis of Aβ toxicity considers the disruption of Na^+/K^+ ATPase by Aβ that leads to elevated Ca^{2+} influx and neurodegeneration. Low concentration of Zn may reduce the toxicity of Aβ by enhancing Na^+/K^+ ATPase enzyme activity (Lovell et al., 1999). 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 (Cuajungco et al., 2000).

1.2.6 Proteins which regulate Zn homeostasis

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) (Cousins et al., 2006). 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 (Cousins et al., 2006). 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) (Kagi and Schaffer, 1988). The role of MTs is to protect cells from Zn deficiency or toxicity (Ebadi et al., 1995). MTs are ubiquitously expressed and their levels are particularly high in parenchymal cells of the intestine, pancreas, kidney, and liver (Cousins et al., 2006). To date, four MTs have been characterized. MT I and MT II are present in most mammalian organs including the brain (Ebadi et al., 1995). MT III was found predominantly in the brain (Palmiter et al.,

1992), whereas MT IV is in epithelial cells (Quaife et al., 1994). 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 (Cousins et al., 2006). 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. (Gaither and Eide, 2000, 2001). Mammalian ZIPs are found in various organs. mRNA of ZIP1 is ubiquitously expressed (Gaither and Eide, 2001), whereas ZIP2 is present only in the spleen, small intestine, and bone marrow (Gaither and Eide, 2000). ZIP3 expression is also high in the spleen and bone marrow (Gaither and Eide, 2000), whereas ZIP4 expression was found in the small intestine and kidney (Wang et al., 2002b). A mutation in the human ZIP4 gene, responsible for intestinal absorption of zinc, has been discovered in Zn metabolism disorder acrodermatitis enteropathica, which was mentioned earlier (Wang et al., 2002b). Human ZIP5 expression is high in the intestines, liver, kidney, and pancreas (Wang et al., 2004a) whereas ZIP6 is expressed in the prostate and placenta (Taylor et al., 2003). 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 (Liuzzi and Cousins, 2004).

1.2.7 Zn transporter (ZnT) proteins in AD

Sequestration of Zn from the cytosol of the cell to the extracellular space or intracellular compartments is regulated by Zn transporters (ZnTs). Previous studies of Zn neurotoxicity and Zn alterations in AD brain provide the basis for our study of Zn transport and ZnT proteins.

In 1995 Palmiter and Findley discovered the first mammalian Zn transporter (ZnT-1) (Palmiter and Findley, 1995). Since that initial discovery nine ZnTs have been

described, Figure 1.4 shows amino-acid similarity of ZnTs. Most ZnTs have six transmembrane domains and histidine rich loops which connect these domains (Palmiter and Huang, 2004) (Figure 1.5). 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.

Immunohistocytochemistry shows ZnT-1 is located at the plasma membrane, the protein is responsible for Zn export from the cytosol to the extracellular space during periods of elevated cytosolic Zn (Palmiter and Findley, 1995) (Figure 1.6). ZnT-1 can be regulated by dietary Zn (Liuzzi and Cousins, 2004). 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 (Sekler et al., 2002). Our previous study of ZnT-1 showed a significant decrease in hippocampus and parahippocampal gyrus (HPG) of mild cognitive impairment (MCI), but a significant elevation in HPG of early AD (EAD) and late AD (LAD) subjects (Lovell et al., 2006).

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 (Palmiter and Findley, 1995). In contrast, Takeda et. al reported rats on a Zn deficient diet had increased levels of Zn in the brain (Takeda, 2000), supporting the idea proposed by Chowanadisai et al. that low systemic Zn may lead to decreased ZnT-1 levels to maintain Zn brain levels (Chowanadisai et al., 2005). 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 (Ohana et al., 2006).

In 1996 Palmiter and colleagues isolated ZnT-2 from a rat kidney cDNA expression library and characterized this transporter (Palmiter et al., 1996a). 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 (Palmiter et al., 1996a), (Figure 1.6). 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 (Liuzzi et al., 2001).

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% (Figure 1.4) (Cousins et al., 2006). ZnT-2 mRNA is barely detectable in mouse brain by reverse transcriptase polymerase chain reaction (RT-PCR) analysis (Palmiter et al., 1996a; Palmiter et al., 1996b).

ZnT-4 is located in lysosomal or/and endosomal compartments and functions to sequester Zn in these compartments (Huang and Gitschier, 1997), (Figure 1.6). ZnT-4 shares 67% and 62% homology with ZnT-2 and ZnT-3 respectively (Figure 1.4) (McMahon and Cousins, 1998). 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 (Huang and Gitschier, 1997). 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 (McMahon and Cousins, 1998). In our study of human brain we detected elevated levels of ZnT-4 protein in HPG of EAD and LAD subjects (Smith et al., 2006).

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 (Huang et al., 2002) (Figure 1.6). 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 (Huang et al., 2002). 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 SMTG of LAD and HPG of EAD subjects compared to NC subjects (Smith et al., 2006). Confocal microscopy experiments revealed that locations of ZnT-6 in MCI brain correlate with the sites of early formation of neurofibrillary tangles (NFTs) (Lovell et al., 2006). 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 (Lovell et al., 2006).

Overall, a large body of literature describes how ZnTs influence mammalian cellular metabolism, however, only a few studies have looked at ZnTs in AD brain. Clearly, additional studies of ZnTs are needed to understand the mechanisms of Zn alterations in AD.

1.3 The role of amyloid beta peptide (Aβ) in AD

1.3.1 Production of Aβ

Senile plaques (SP), one of the pathological hallmarks of AD, are mainly composed of amyloid beta peptide (Aβ) (Wilquet and De Strooper, 2004). This relatively short peptide (~ 4 kDa) results from the proteolytic cleavage of 110−120 kDa amyloid precursor protein (APP). α-, β-, and γ-secretases are involved in the cleavage of APP. Figure 1.7 shows Aβ processing from APP (Wilquet and De Strooper, 2004). APP is a transmembrane protein with intracellular C- and extracellular N-termini. Cleavage of the majority of APP by α-secretase leads to the production of soluble secreted APP, and is usually considered the non-amyloidogenic pathway. In contrast, cleavage by β-secretase leads to the formation of Aβ. Both α-secretase and β-secretase cleave the extracellular domain of APP, resulting in neurotrophic APPsα and APPsβ respectively. Further cleavage of the transmembrane domain of the APP by γ-secretase produces carboxy terminal fragments (α-CTF and β-CTF), releasing the p3 and the Aβ peptide, respectively, into the extracellular space and the APP intracellular domain (AICD) into the cytoplasm.

It has been a while since APP and its proteolytic products were discovered, however physiological functions of these species remain unclear. Studies by Ninomiya et al. showed the secreted ectodomain of APP (APPs) promotes growth of fibroblasts (Ninomiya et al., 1993). Another study by Caille et al. suggested the subventricular zone, the largest neurogenic area of the adult brain, may be a major APPs binding site. APP_{695} is the predominant isoform of APP in the brain. APP_{695} exerted growth-promoting activities on adult progenitor cells in the subventricular zone but not in the dentate gyrus

of the hippocampus (Caille et al., 2004). Possible ligands for APP_{695} include F-spondin (Ho and Sudhof, 2004), a secreted glycoprotein thought to play a role in neuronal regeneration. In cell cultures F-spondin binding may be involved in the regulation of APP cleavage though the inhibition of β-secretase (Ho and Sudhof, 2004). In addition, Pietrzik et al. showed interactions between cytoplasmic domains of APP and the large low-density lipoprotein (LDL) receptor-related protein (LRP), and demonstrated that this binding increases APP proteolytic processing. LRP, a transmembrane receptor, localized predominantly in hepatocytes, fibroblasts, and neurons, is involved in multiple biological processes acting as an endocytic receptor and signaling molecule (Kinoshita et al., 2003a). Both proteins, LRP and APP, bind the adaptor protein FE65, suggesting physiological link between the two (Pietrzik et al., 2004). Kinoshita et al. proposed that the LRP intracellular domain negatively regulates APP gene transcription by the AICD/FE65/Tip60 complex, where Tip60 is a transcription modulator (Kinoshita et al., 2003a). However, an exact mechanism that regulates APP receptor-associated activity *in vivo* is not clear. Little is known about the strongly hydrophobic fragment p3 (Wilquet and De Strooper, 2004). In contrast, the role of the AICD fragment has received more attention. It is thought to function in nuclear signaling due to its similarity to Notch, which influences cell fate in the developing nervous system (Gaiano and Fishell, 2002), and APP processing (Herreman et al., 1999).

1.3.2 Subcellular locations of β- and γ-secretases

β-Secretase is a membrane-tethered enzyme in the pepsin family of aspartyl proteases, primarily expressed in the brain (Cole and Vassar, 2008). The early work by Sambamurti et al. suggested that APP is cleaved by β-secretase in the trans-Golgi network (TGN) (Sambamurti et al., 1992). The study by Vassar et al. described a substantial proportion of β-secretase immunostaining in the Golgi and in endosomes with only a small amount of staining in the endoplasmic reticulum (ER) and lysosomes (Vassar et al., 1999). Later Hyman and colleagues analyzed subcellular localization of interactions between APP and β-secretase by double immunofluorescence and a fluorescence resonance energy transfer (FRET) in H4 neuroglioma cells co-transfected with APP and β secretase (Kinoshita et al., 2003b). They reported that the majority of cell

surface APP and β-secretase were internalized after 15 minutes, but they remained strongly co-localized together in the early endosomal compartment, where FRET analysis demonstrated a continued close interaction (Kinoshita et al., 2003b). The most current view on β-secretase and its subcellular localization is presented in the review by Hu et al., suggesting β-secretase has an acidic pH optimum and is localized within intracellular compartments of the secretory pathway including both the Golgi and endosomes (Hu et al., 2007).

In contrast to β-secretase, the ubiquitously expressed γ-secretase is a complex of four different integral membrane proteins essential to the protease: presenilin (PS), Nicastrin, Aph-1, and Pen-2 (Wolfe, 2006). As mentioned earlier, two homologous presenilins (PS1 and PS2) are genetically linked to numerous cases of familial AD and are apparently of exceptional importance for γ-secretase cleavage. Reports on subcellular location of APP cleavage by γ-secretase are controversial (Sudoh et al., 2000; Baulac et al., 2003; Pasternak et al., 2004; Vetrivel et al., 2004). Sudoh et al. showed that γ-secretase cleavage of APP occurs in the Golgi compartment and the TGN (Sudoh et al., 2000). That observation was confirmed in more recent work by Baulac et al. (Baulac et al., 2003). They used subcellular fractionation of membrane vesicles and subsequent coimmunoprecipitation along with the immunofluorescent staining of the individual γ-secretase components, and concluded the proteolytically active γ-secretase complex is located in the Golgi/TGN (Baulac et al., 2003). Additional incubation of the Golgi/TGN-enriched vesicles resulted in *de novo* generation of Aβ protein and APP intracellular domain (Baulac et al., 2003). In contrast, Pasternak et al. showed that in neurons and astrocytes, γ-secretase cleaves APP in lysosomes (Pasternak et al., 2004), and Vetrivel et al. reported the localization of γ-secretase in post-Golgi and endosomes (Vetrivel et al., 2004).

Intracellular Aβ has also been found in multiple subcellular locations (Petanceska et al., 2000; Cataldo et al., 2004). The study by Petanceska et al. showed the highest levels of Aβ colocalized with rab8, a marker for TGN-to-plasma membrane transport vesicles (Petanceska et al., 2000).The recent study by Cataldo et al. showed that early endosomes are the possible site of APP processing, and intracellular Aβ is localized to rab5-positive endosomes in neurons from AD brains (Cataldo et al., 2004).

To conclude, reports on subcellular locations of β- and γ-secretases as well as Aβ production site have been conflicting. We believe that secretases cleave APP to Aβ in acidic organelles which may be endosomes, lysosomes and/or Golgi/TGN.

1.3.3 Amyloid hypothesis of AD

According to the amyloid hypothesis, AD is caused by the progressive accumulation and deposition of neurotoxic Aβ in senile plaques and aggregates in brain (Marcello et al., 2008). Although the exact mechanism of Aβ neurotoxicity remains unclear several hypotheses have been proposed (Small et al., 2001; Walsh et al., 2002; Lu et al., 2003; Glabe, 2006). For several years, soluble Aβ was considered less toxic than fibrillar Aβ which is the main component of plaques in AD brain. Toxicity of fibrillar Aβ was attributed to nerve-cell death induced by Aβ deposits (Simmons et al., 1994; Howlett et al., 1995). Thus, Aβ accumulation was thought to be the primary event in AD pathogenesis.

The most recent opinion considers an alternative mechanism for memory loss and focuses on soluble Aβ oligomers as the primary pathogenic structures rather than mature Aβ fibrils (Glabe, 2006). Oligomers of Aβ have the ability to permeabilize a cellular membrane and initiate a core sequence of pathological events such as increased production of oxidative species, calcium dysregulation, and mitochondrial dysfunction leading to cell dysfunction and death. In addition, the study by Wang et al. showed that Aβ oligomers inhibit long-term potentiation, a classic experimental paradigm for memory and synaptic plasticity (Wang et al., 2002a).

Proteolysis of APP by γ-secretase is heterogeneous: most full-length Aβ species produced are 40-residue peptides ($A\beta_{40}$), whereas a small proportion is a 42-residues variant ($A\beta_{42}$) (Marcello et al., 2008). Under physiological conditions the ratio of $A\beta_{40}$ to $A\beta_{42}$ is 1:10. The study by Jarrett et al showed that $A\beta_{42}$ oligomers can play the role of seeds in the aggregation of $A\beta_{40}$ (Jarrett and Lansbury, 1993). $A\beta_{42}$, the predominant form in diffuse and neuritic plaques in AD brain, is believed to be more important in AD pathogenesis due to its aggregative ability and higher neurotoxicity compared to $A\beta_{40}$ which is mainly found in vascular deposits (Irie et al., 2005).

1.4 Statements of research projects

1.4.1 Alterations in levels of ZnT-1, ZnT-4 and ZnT-6 in the brain of subjects with preclinical Alzheimer's disease (PCAD)

As discussed earlier, increasing evidence suggests that the etiology of Alzheimer's disease (AD) may involve disruptions of zinc (Zn) homeostasis. Our previous studies demonstrate alterations of Zn transporter proteins ZnT-1, ZnT-4, and ZnT-6 in vulnerable brain regions of subjects with mild cognitive impairment (MCI), early and late stage AD (Lovell et al., 2006; Lovell et al., 2006; Smith et al., 2006). ZnT-1 exports Zn from the cytosol to extracellular compartments, ZnT-4 transports Zn from the cytosol to lysosomes and endosomes, and ZnT-6 sequesters Zn in the trans-Golgi network. A preclinical stage of AD (PCAD) has been described in which subjects show no overt clinical manifestations of AD but demonstrate significant AD pathology at autopsy (Lange et al., 2002; Knopman et al., 2003; Galvin et al., 2005).

To determine if alterations of ZnT proteins occur in PCAD we measured ZnT-1, ZnT-4, and ZnT-6 in the hippocampus/parahippocampal gyrus (HPG) and cerebellum (CER) of seven PCAD subjects and seven age matched normal control (NC) subjects using Western blot analysis and custom protein specific antibodies.

1.4.2 ZnT-2 levels in the brain of subjects with preclinical Alzheimer's disease (PCAD), mild cognitive impairment (MCI), early (EAD) and late-stage Alzheimer's disease (LAD)

This is the first study of ZnT-2 in the human brain. The function of ZnT-2 is to sequester Zn from cytosol to lysosomal and endosomal compartments (Palmiter et al., 1996a). We detected mRNA of ZnT-2 in human brain via reverse transcriptase polymerase chain reaction (RT-PCR) and quantified protein levels of ZnT-2 via Western blot analysis in three brain regions of patients with different stages of AD.

Based on our results and previous studies of ZnTs in AD brain, *we hypothesized that ZnT-2 protein levels may be altered in brains of subjects with AD* (Lovell et al., 2006; Lovell et al., 2006; Smith et al., 2006). In the present study we measured ZnT-2 in the hippocampus/parahippocampal gyrus (HPG), superior and middle temporal gyrus (SMTG) and cerebellum (CER) of five PCAD, five mild cognitive impairment (MCI), five early (EAD), six late AD (LAD) and four age matched NC subjects via Western blot analysis.

We believe the location of ZnT-2 indicates its imperative role in the formation of amyloid beta peptide (Aβ) which forms senile plaques in AD brain. Recently it was discovered by Kinoshita et al. that β-secretase cleaves APP in intracellular acidic compartments (Kinoshita et al., 2003b). One of the requirements of the amyloid precursor protein (APP) cleavage by β-secretase is the low pH (Wilquet and De Strooper, 2004). The presence of Zn may shift the equilibrium to a more acidic pH. Therefore, increased concentrations of Zn may provide the optimum conditions for β-secretase processing and contribute to the production of toxic Aβ. We speculate that altered ZnT-2 levels in AD vulnerable brain regions may lead to Zn accumulation in endosomal/lysosomal compartments and contribute to formation of Aβ.

1.4.3 A role for ZnT-1, ZnT-2, ZnT-4 and ZnT-6 in amyloid beta (Aβ) peptide processing

Our previous studies and results from projects described in this dissertation show alterations of ZnT-1, ZnT-2, ZnT-4 and ZnT-6 in brains of subjects with PCAD, MCI, EAD and LAD compared to age matched NC subjects.

Because alterations of Zn have been associated with Aβ processing and senile plaque formation 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 amyloid precursor protein (APP), treated with siRNAs against each ZnT.

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 intracellular compartments (endosomes/lysosomes/Golgi/trans Golgi network) (Kinoshita et al., 2003b; Cataldo et al., 2004; Pasternak et al., 2004). By reducing levels of ZnTs using siRNAs, the amount of Zn in intracellular organelles, where Aβ processing occurs, should be decreased compared to levels in control cells leading to diminished cleavage of APP by β- and γ-secretases and decreased levels of Aβ.

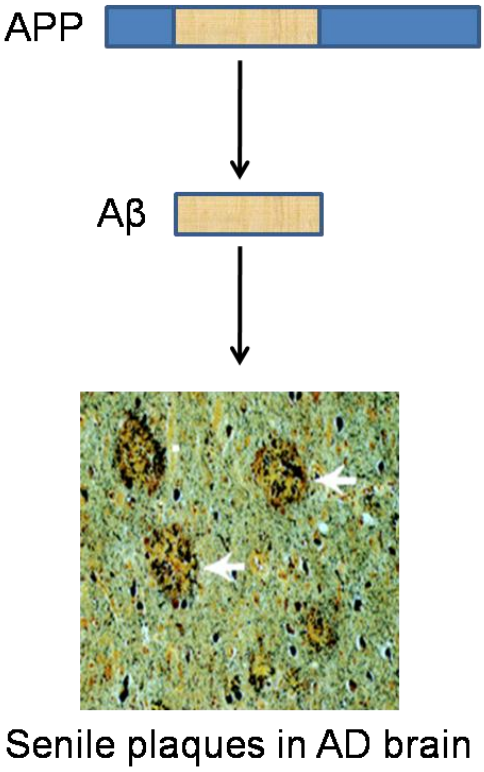

Figure 1.1. Scheme of senile plaque formation. Senile plaques in AD brain are shown with white arrows (adapted from Lovell et al., 1998).

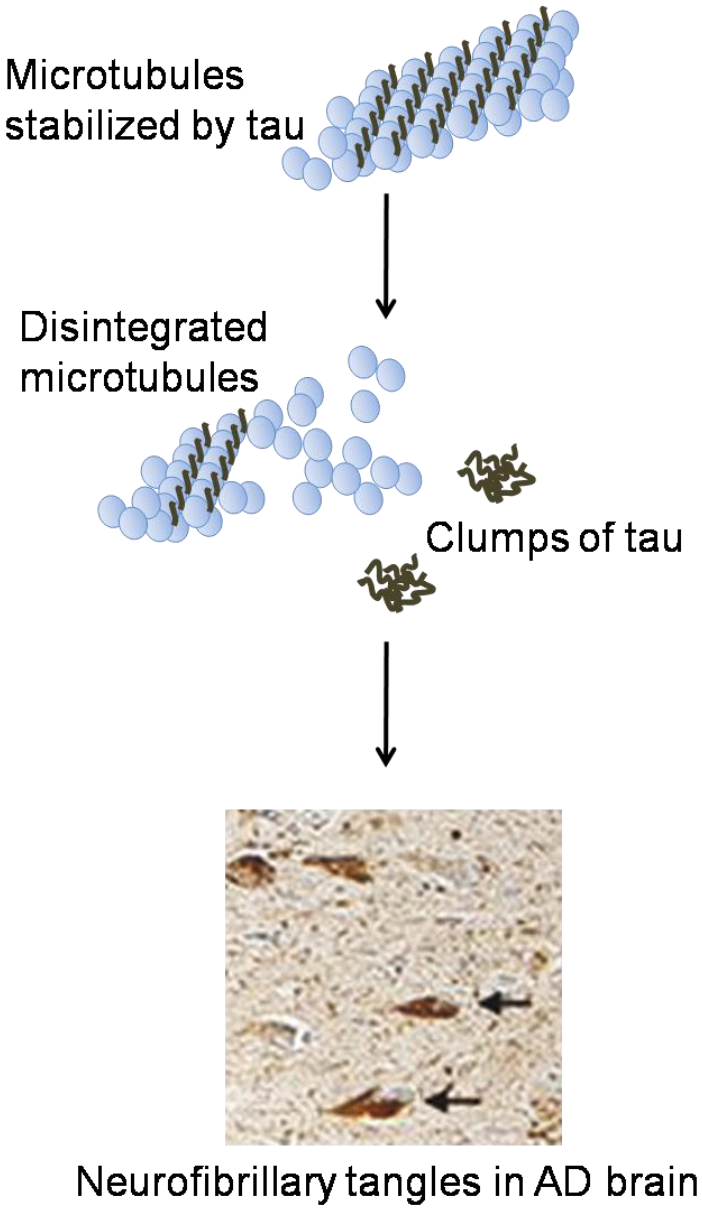

Figure 1.2. Scheme of neurofibrillary tangle formation. Neurofibrillary tangles in AD brain are shown with the black arrows (adapted from Lovell et al., 2004).

Table 1.1. Clinical and histopathological criteria for stages of Alzheimer's disease (AD)

Stage of Alzheimer's Disease (AD)	Clinical criteria	Histopathological criteria
Preclinical Alzheimer's Disease (PCAD)*	✓ Normal range ✓ CDR 0	✓ NIA-RI intermediate or high likelihood ✓ Braak III-V
Mild Cognitive Impairment (MCI) (Petersen et al., 1999; Petersen, 2007)	✓ Memory complains ✓ Memory impairment ✓ Intact general cognitive function ✓ Intact ADLs ✓ CDR < 0.5	✓ NIA-RI low likelihood ✓ Significant increase in SPs in neocortical regions and NFTs in entorhinal cortex, hippocampus and amygdala ✓ Braak III - IV
Early Alzheimer's Disease (EAD) (Anonymous, 1997; Morris and Price, 2001; Price, 2003)	✓ Declines in one or more areas of cognition in addition to memory ✓ CDR 0.5-1.0 ✓ No other causes of dementia ✓ Impaired ADLs	✓ NIA-RI high likelihood ✓ Braak V
Late Alzheimer's Disease (LAD) (Anonymous, 1997; Morris and Price, 2001; Price, 2003)	✓ Cognitive deficit in all domains ✓ Language impairment ✓ CDR 1.0-3.0 ✓ Require special care	✓ NIA-RI high likelihood ✓ Braak VI

* Criteria by University of Kentucky Alzheimer's Disease Center

ADL- activities of daily living
CDR - Clinical dementia rating
CERAD – The Consortium to Establish a Registry for Alzheimer's Disease
NFT – neurofibrillary tangles
NIA-RI – National Institute on Aging– Reagan criteria
SP – senile plaques

Figure 1.3. Protein sequences of human and rat $A\beta_{42}$. Amino acids shown with red are different in human and rat $A\beta_{42}$ sequences. Histidines shown with blue are potential binding sites for Zn in human $A\beta_{42}$ (adapted from Bush et al., 1994).

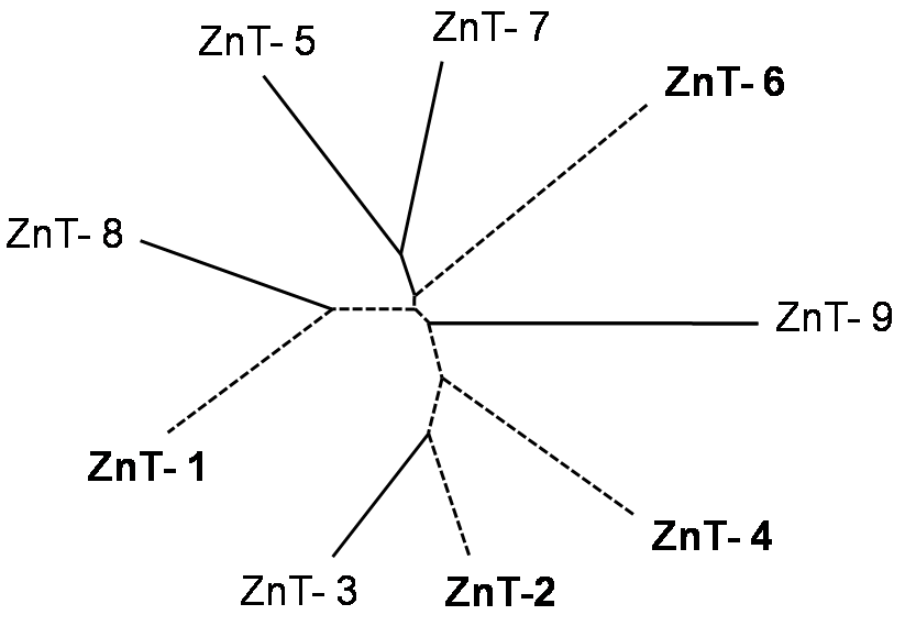

Figure 1.4. The above dendrogram shows amino-acid sequence similarity between ZnT proteins (adapted from Palmiter and Huang, 2004). The more branches shared by ZnTs, the greater similarity they have in amino-acid sequence. Relationships between ZnTs analyzed in our studies are indicated with dotted lines.

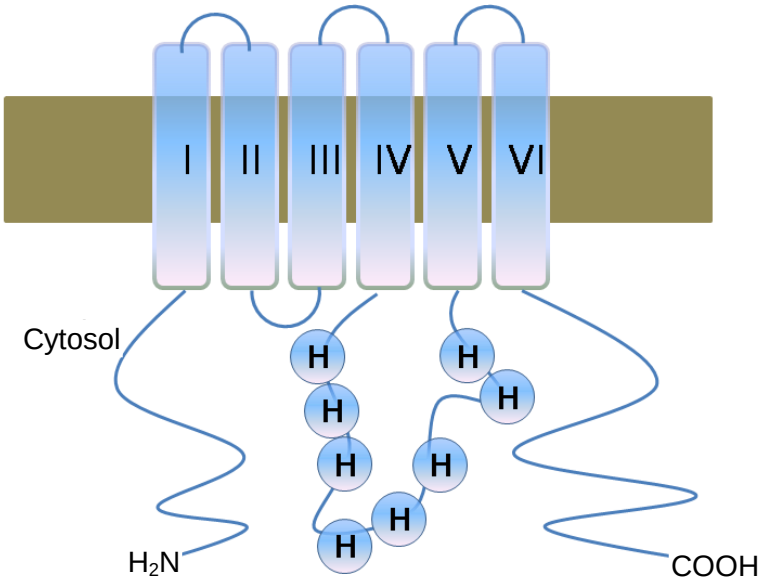

Figure 1.5. Predicted topology of ZnTs, H- histidine (adapted from Harris, 2002).

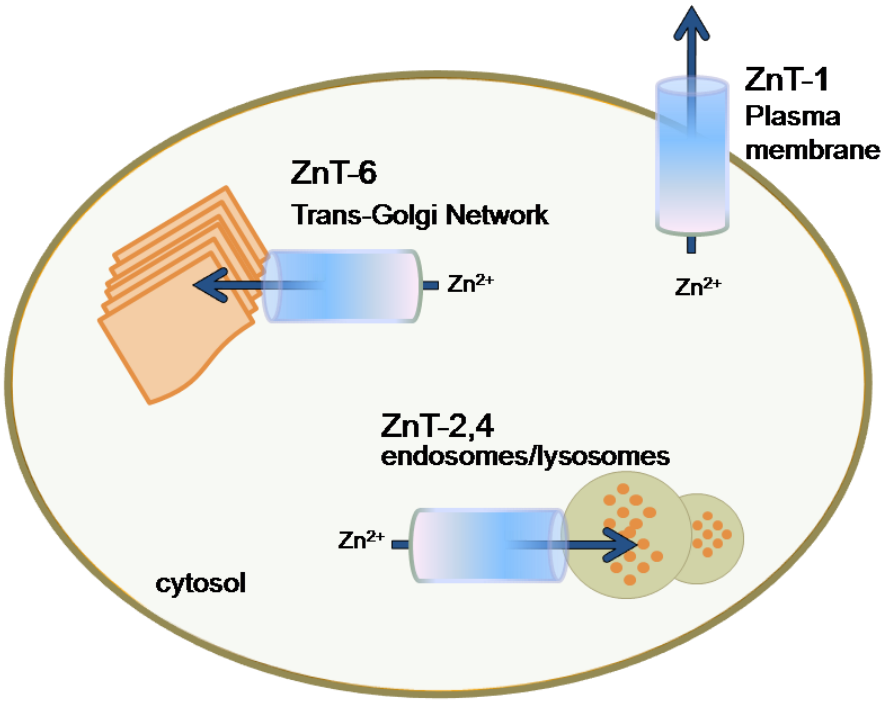

Figure 1.6. Cellular location of ZnTs (adapted from Palmiter and Huang, 2004).

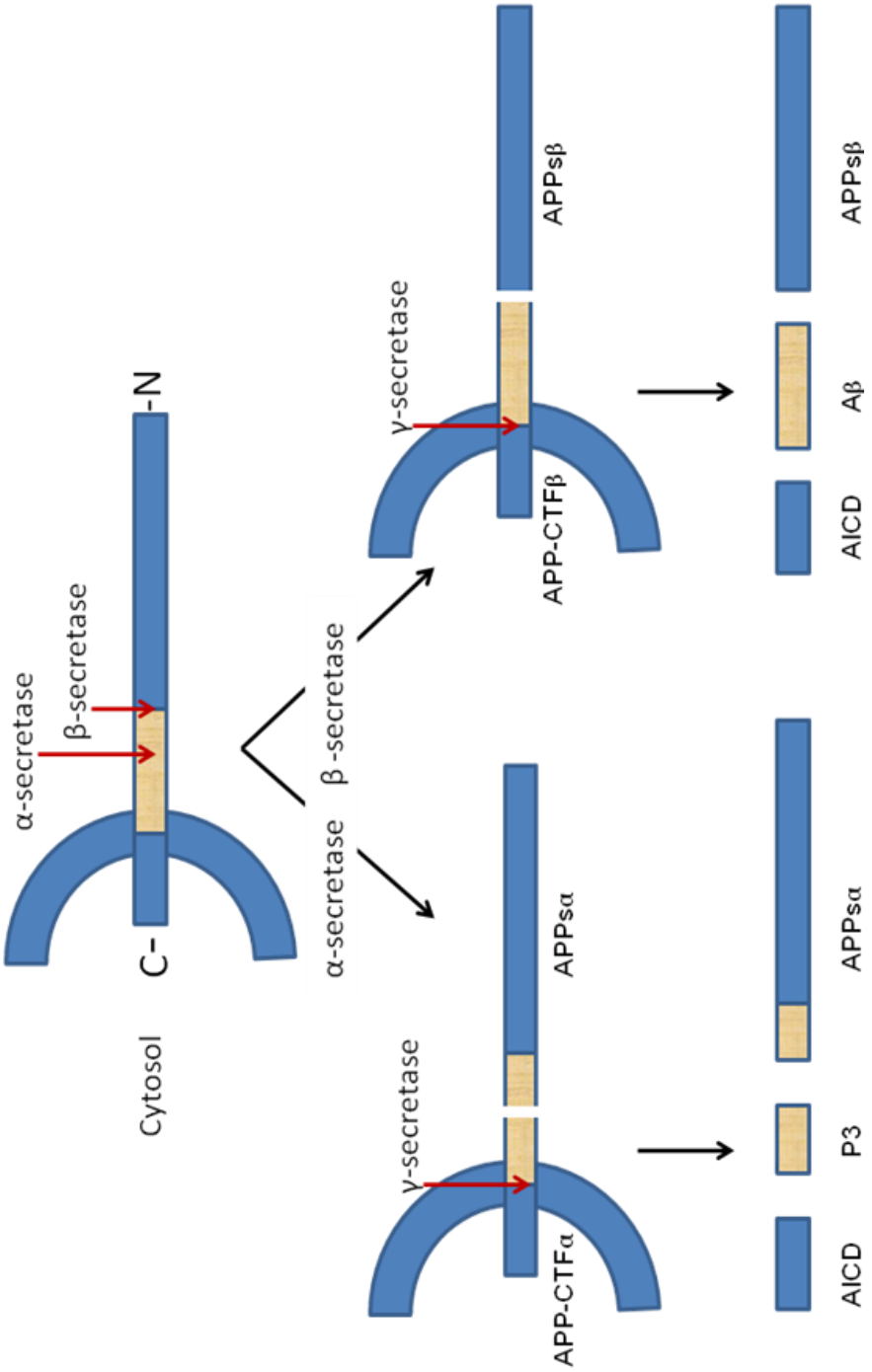

Figure 1.7. Schematic of the Aβ formation from APP (adapted from Wilquet et al., 2004).

CHAPTER TWO

Materials and methods

2.1 Materials

2.1.1 Brain specimen sampling

Tissue specimens from the hippocampus and parahippocampal gyrus (HPG) and cerebellum (CER) of short postmortem interval (PMI) autopsies of seven PCAD and seven age matched normal control (NC) subjects (PCAD project); five PCAD (3 men, 2 women), five MCI (2 men, 3 women), five EAD (2 men, 3 women), six LAD (4 men, 2 women) and four age matched NC subjects (2 men, 2 women) (ZnT-2 project) were obtained through the neuropathology core of the University of Kentucky Alzheimer's Disease Center (UK-ADC). These samples were immediately frozen at autopsy in liquid nitrogen and stored at –80^{o}C until used for analysis.

PCAD and NC subjects were followed longitudinally in the UK-ADC Normal Control Clinic and had neuropsychologic testing, physical and neurological examinations annually. All NC subjects had neuropsychological test scores in the normal range and showed no evidence of memory decline. Although there are not precise criteria for the diagnosis of PCAD, the UK-ADC tentatively describes PCAD subjects as those with sufficient AD pathologic alterations to meet intermediate or high NIA-Reagan Institute (NIA-RI) criteria with Braak scores of III-V, moderate or frequent neuritic plaque scores according to Consortium to Establish a Registry for AD (CERAD), and antemortem psychometric test scores in the normal range when corrected for age and education. Recent studies by Galvin et al. (Galvin et al., 2005) and Knopman et al. (Knopman et al., 2003) suggest ~ 30% of control subjects had high or intermediate NIA-RI changes but were not demented and were likely PCAD subjects. MCI subjects in our studies were normal when they enrolled into the UK-ADC longitudinal study, but developed MCI during followup. Clinical criteria for diagnosis of amnesic MCI are those of Petersen et al. (Petersen et al., 1999) which include 1) memory complaints corroborated by an informant, 2) abnormal memory impairment for age and education, 3) normal general cognitive function, 4) intact activities of daily living, and 5) the subject does not meet criteria for dementia. EAD and LAD subjects met both standard clinical criteria for

probable AD and standard histopathological criteria for the diagnosis of AD (McKhann et al., 1984; Mirra et al., 1991). The clinical criteria for EAD are 1) a decline in cognitive function from a previous higher level, 2) declines in one or more areas of cognition in addition to memory, 3) a clinical dementia rating scale score of 0.5–1, and 4) a clinical examination that excluded other causes of dementia. The clinical criteria for LAD are 1) cognitive deficit in all domains, 2) language impairment, 3) a clinical dementia rating scale score of 1.0-3.0, and 4) patient requires special care.

Neuropathological examination of subjects in our studies was carried out by the Neuropathology Core of the UK-ADC. All subjects had neuropathological examination of multiple sections of neocortex, hippocampus, entorhinal cortex, amygdala, basal ganglia, nucleus basalis of Meynert, midbrain, pons, medulla, and CER using the modified Bielschowsky stain, hematoxylin and eosin stains, and Aβ and α-synuclein immunostains. Braak staging scores (Braak and Braak, 1991) were determined using the Gallyas stain on sections of entorhinal cortex, hippocampus, and amygdala and the Bielschowsky stain on neocortex. Histopathologic examination of NC subjects showed only age-associated changes and Braak staging scores of I to III. Four NC subjects did not meet CERAD criteria for histopathologic diagnosis of AD and two NC subjects met CERAD criteria for probable AD. All NC subjects met NIA-RI low likehood criteria for histopathologic diagnosis of AD. Three PCAD subjects did not meet CERAD criteria for the diagnosis of AD, three PCAD subjects met CERAD criteria for probable AD and two PCAD subjects had plaque pathology which corresponds to definite diagnosis of AD according to CERAD criteria. All PCAD subjects met intermediate or high NIA-RI criteria (Braak III). The difference between MCI and NC patients was a significant increase in neuritic plaque density in neocortical regions and a significant increase in neurofibrillary tangle density in entorhinal cortex, hippocampus, and amygdala in MCI patients (Markesbery et al., 2005). The Braak staging scores of MCI subjects ranged from III to IV. EAD subjects met high likelihood criteria for the histopathological diagnosis of AD with a Braak staging score of V. All AD subjects had Braak staging scores VI.

2.1.2 H4-APP cell cultures

To examine the role of Zn sequestration mediated by ZnT-1, ZnT-2, ZnT-4 and ZnT-6 in Aβ processing, we used H4 human neuroglioma cell lines transfected to overexpress the amyloid precursor protein (APP) (H4-APP cells) (Krex et al., 2001) treated with siRNAs specific to each protein of interest (Ambion, Austin, TX). Cells were maintained in OptiMEM (Invitrogen) with 10% fetal bovine serum (FBS) and hygromicin B (0.2 mg per ml) and were split 1:2 every two days.

2.1.3 Antibodies

Custom rabbit anti-ZnT-1, anti-ZnT-4 and anti-ZnT-6 polyclonal antibodies were prepared by Chemicon (Temecula, CA, USA). The ZnT-1 antibody was produced against a KLH conjugated peptide (GTRPQVSHGKE) that corresponds to amino acids 448-458 of the carboxyl terminus of rat ZnT-1 protein and shows significant homology to human ZnT-1. The peptides used in the production of the other two antibodies were unique to the sequence of each human protein: ZnT-4 (DSCDNCSKQPEILKQRKV) and ZnT-6 (QGLRTGFTYIPSR). Rabbit anti-GAPDH was used as a protein loading control (Santa Cruz, CA, USA). The goat anti-ZnT-2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase conjugated goat anti-rabbit and rabbit anti-goat secondary antibodies were purchased from Amersham Biosciences (Piscataway, NJ).

2.2 Methods

2.2.1 Tissue processing

Tissue specimens were homogenized in 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) containing 137 mM NaCl, 4.6 mM KCl, 0.6 mM $MgSO_4$, 0.7 µg/ml pepstatin A, 0.5 µg/ml leupeptin, 0.5 µg/ml aprotinin, and 40 µg/ml phenylmethyl sulphonyl fluoride, using a Dounce homogenizer on ice. The resulting homogenate was centrifuged at 800 x *g* and 4°C to pellet cellular debris. The supernatant from the initial slow speed spin was centrifuged at 100,000 x g for 1 hr and 4°C to pellet cell membranes containing ZnT-1. The supernatant from the high speed spin

containing cellular organelles was removed and used for ZnT-2, ZnT-4 and ZnT-6 measurements. The pellet containing cell membranes was rinsed twice with HEPES buffer, resuspended in HEPES buffer and rehomogenized. Protein concentrations were determined for the pellet and supernatant using the Pierce bicinchoninic acid (BCA) protein assay (Sigma, St. Louis, MO).

2.2.2 Reverse transcriptase polymerase chain reaction (RT-PCR) amplification of ZnT-2 mRNA

To verify the presence of ZnT-2 in human brain, total RNA was isolated from representative tissue specimens using an RNeasy Mini Kit from QIAGEN (Valencia, CA). For reverse transcriptase polymerase chain reaction (RT-PCR) amplification we used SuperScript One-Step RT-PCR Systems from Invitrogen (Carlsbad, CA). Complementary DNA (cDNA) was prepared from 300 ng of total RNA. We used primer sequences similar to those used for mouse ZnT-2 (Palmiter et al., 1996b). Primers were synthesized by Integrated DNA technologies (IDT, Coralville, IA). The RT-PCR mix concentrations of both primers were 0.2 μM. cDNA were synthesized and amplified by the following RT-PCR procedure: 1) 50°C for 30 min; 2) 94°C for 2 min; 3) 38 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec; 4) 72°C for 10 min 5) incubation at 4°C. RT-PCR products were separated on 1.2% agarose gels and bands were visualized with ethidium bromide. Identity of the RT-PCR product was confirmed by sequencing (UC Davis Sequencing Facility, Davis, CA). Sequences were aligned using GenBank and BLAST tools.

2.2.3 Induction of ZnTs in H4-APP cell cultures

To determine the temporal profile of ZnT induction, H4-APP cells were treated with 50 μM Zn for 0, 0.5, 2, 4, 8 and 16 hours. Protein levels of each ZnT were determined at each time point using Western blot analysis and antibodies specific for each protein.

2.2.4 siRNA transfection of H4-APP cell cultures

H4-APP cells were trypsinized and resuspended in OptiMEM at 37°C. Cultures were transfected with siRNA by mixing 30 µl NeoFX transfection reagent (Ambion, Austin, TX) with 600 µl OptiMEM and incubating 10 min at room temperature. Annealed siRNA was mixed with 600 µl OptiMEM to a final concentration of 40 nM (in each well on the 6 well plate) at room temperature, added to the NeoFX solution and incubated 10 min at room temperature. 200 µl of the mixture was then added to each well of a 6 well plate and cultures added at a density of 2.5 x 10^5 cell/well in 2.3 ml OptiMEM. For initial experiments, three *Silencer* pre-designed siRNA sequences for each ZnT were purchased from Ambion and tested for effectiveness at diminishing ZnT expression. Of three sequences tested for each ZnT, at least one led to a significant ($P < 0.05$) decrease in corresponding protein levels following 24 h transfection at 37°C. Effective siRNAs had the following sequences: 5'-CCU AUC CAU UAC UUA AGG ATT-3' (sense sequence for ZnT-1), 5'-UCC UUA AGU AAU GGA UAG GTT-3' (antisense sequence for ZnT-1); 5'-GCG GGU AUA AAG CUA GUG UTT-3' (sense sequence for ZnT-2), 5'-ACA CUA GCU UUA UAC CCG CTG-3' (antisense sequence for ZnT-2); 5'-CGU AAC CAU GGG CAG GAU ATT-3' (sense sequence for ZnT-4), 5'-UAU CCU GCC CAU GGU UAC GTT-3' (antisense sequence for ZnT-4); 5'-CGA UGC UUU CUA UUC GGA ATT-3' (sense sequence for ZnT-6), 5'-UUC CGA AUA GAA AGC AUC GTG-3' (antisense sequence for ZnT-6). Non gene-specific negative controls were composed of 19 bp scrambled sequences with 3'dT overhangs that had no significant homology to any known gene sequence from mouse, rat or human and showed no gross changes in expression of GAPDH or cyclophilin suggesting a lack of nonspecific effects on gene expression. For one siRNA treatment we used three of 6 well plates and each siRNA experiment was repeated three times.

2.2.5 Treatment of siRNA transfected cells

After a 24 h transfection period cultures were treated with 50 µM $ZnSO_4$ in OptiMEM for 16 h at 37°C (except for ZnT-1 siRNA experiments for which 8 h treatment times were used at 37°C). 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium

bromide (MTT) assay was used as a measure of cell viability. A treatment time of 16 h was chosen because there was no significant ($P < 0.05$) reduction in MTT, compared to controls (100 ± 1.6% total MTT), whereas longer treatment times led to significant ($P < 0.05$) decreases in MTT release and loss of membrane integrity (83.7 ± 2.5% total MTT at 24 h). MTT viability assays were performed as previously described (Mosmann, 1983). Following treatment, cells were collected for protein analysis via Western blot analysis and cell media was collected for measurements of Aβ levels via enzyme-linked immunosorbent assays (ELISA).

2.2.6 Aβ enzyme-linked immunosorbent assay (ELISA)

Levels of Aβ production by H4-APP cells were measured in medium from cultures treated with ZnT siRNAs and Zn. Medium was collected from each well and EDTA added to a final concentration of 2 mM to prevent proteolytic degradation of Aβ. Aβ ELISAs were carried out as previously described (Liu et al., 2007). Briefly, total Aβ is captured from conditioned medium with a monoclonal antibody (Ab9) that recognizes $Aβ_{1-16}$ and detects both monomeric and aggregated human Aβ but does not bind rodent Aβ. $Aβ_{1-40}$ and $Aβ_{1-42}$ are then detected with HRP conjugated monoclonal antibodies that recognize $Aβ_{38-40}$ (Ab1.3.1) and $Aβ_{38-42}$ (Ab 2.3.1).

2.2.7 Western blot analysis

Protein samples (20 μg) were separated by electrophoresis on 4-15% gradient sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in 5% dry milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TTBS) solution for 1 hr at room temperature. Primary antibodies were incubated overnight at 4°C. The dilutions of primary antibodies were 1:500 for ZnT-1, ZnT-2, ZnT-6, and 1:1000 for ZnT-4. Membranes were washed with TTBS three times at room temperature and incubated in horseradish peroxidase conjugated goat anti-rabbit secondary antibody (ZnT-1, ZnT-4 and ZnT-6) or rabbit-anti-goat secondary antibody (ZnT-2) for 2 hr at room temperature and washed. Bands were visualized using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA) using manufacturer's instructions. After development, the membranes were stripped and

reprobed for GAPDH (1:5000 dilution) as a protein loading control. Band intensities for ZnT-1, ZnT-2, ZnT-4, and ZnT-6 proteins were quantified using Scion Image Analysis software (Scion, Frederick, MD).

2.2.8 Confocal microscopy and quantitative comparisons

Confocal microscopy and quantitative comparisons of ZnT-1 and ZnT-6 intensities in HPG of PCAD and NC subjects were performed as described previously (Lovell et al., 2006). Briefly, 10 µm sections of paraffin-embedded HPG from PCAD and NC subjects were cut using a Shandon Finesse microtome (Thermo Electron, Waltham, MA), placed on Plus-slides and rehydrated through xylene, descending alcohols, and water. Sections were incubated for 10 minutes in a 0.75 mg/ml trypsin type II (Thermo Electron, Waltham, MA) solution prepared in 150 mM Tris HCl (pH 7.6) containing 3.3 mM $CaCl_2$ at 37°C. The sections were washed three times in phosphate buffered saline (PBS), allowed to air dry and then incubated in 50 mM ammonium chloride at room temperature for 30 minutes.

For confocal microscopy, sections were incubated overnight in a 1:100 dilution of ZnT-1 or ZnT-6 and a 1:100 dilution of MC-1, a conformation-dependent monoclonal antibody that recognizes distinct pathologic confirmations of tau observed only in AD brain and identifies early NFT formation (kindly provided by Dr. Peter Davies). All primary antibody solutions were prepared in TBS containing 1% goat serum (GS) (Invitrogen, Carlsbad, CA). Following thorough rinsing in TBS, the sections were incubated 1 hour with a 1:1000 dilution of Alexa-488 conjugated anti-rabbit and Alexa-673 conjugated anti-mouse secondary antibodies (Molecular Probes, Eugene, OR) prepared in TBS containing 1% GS. After 5 rinses in TBS and distilled/deionized water, the sections were coverslipped using fluorescent anti-fade (Molecular Probes) and imaged using a Leica DM IRBE confocal microscope equipped with argon, krypton and HeNe lasers and a 40X oil objective. Confocal images were captured from a single z plane without optical sectioning. Fluorescent intensities for ZnT-1, ZnT-6 and MC-1 were quantified using Leica software (Leica Microsystems Heidelberg GmbH, Mannheim, Germany).

2.2.9 Statistical analysis

For Western blot analysis of brain samples, individual integrated band densities for the protein of interest were normalized to GAPDH densities. Normalized ZnT values were averaged for NC subjects and each NC and PCAD, MCI, EAD, LAD value was normalized to the mean control levels for each individual gel. Similarly for the Western blot analysis of H4-APP cells, individual integrated band densities normalized to GAPDH were averaged for controls and the intensity of each band was normalized to mean control cell levels for each individual gel. For Aβ ELISA analyses, individual measurements were averaged for controls and each measurement was normalized to mean control levels for each individual experiment. Normalized values were compared using analysis of variance (ANOVA) and the commercially available ABSTAT software (Anderson Bell, Arvada, CO, USA). Results are presented as mean $\pm$ S.E.M (% NC for the human brain studies; % control for the cell culture study) for ZnT-1, ZnT-2, ZnT-4, or ZnT-6 and total Aβ. Results of MC-1, ZnT-1 and ZnT-6 immunohistochemical studies are reported as mean $\pm$ S.E.M (% NC). Correlation analyses of ZnT-1, ZnT-6 and MC-1 immunostaining were performed using ABSTAT. Braak staging scores were compared using nonparametric testing and the Mann-Whitney U test.

CHAPTER THREE

Results

3.1 Alterations in levels of ZnT-1, ZnT-4, and ZnT-6 in the brain of subjects with preclinical Alzheimer's disease (PCAD)

Subject demographic data are shown in Table 3.1. There were no significant differences between PCAD and NC subjects in age or PMI. Median Braak staging scores were significantly higher in PCAD subjects (IV) compared to NC subjects (I). Western blot analysis showed the presence of ZnT-1 by the appearance of a band at ~ 52 kDa. ZnT-4 was observed at approximately 48 kDa and ZnT-6 at 51 kDa. Previous studies show the antibodies are specific for the proteins of interest (Lovell et al., 2006; Lovell et al., 2006; Smith et al., 2006).

Figures 3.1, 3.2, and 3.3 show Western blots and results of quantification of immunostaining intensity for levels of ZnT-1, ZnT-4 and ZnT-6 reported as mean $\pm$ S.E.M. (% of NC) in HPG and CER of PCAD and age matched NC subjects. Levels of ZnT-1 in HPG of PCAD subjects were significantly ($P < 0.05$) lower (62.3 $\pm$ 3.8%; n = 7) compared to NC subjects (100 $\pm$ 8.2%; n = 7) (Figures 3.1 A, B). ZnT-1 levels in CER were not significantly different between PCAD and NC subjects (Figures 3.1 A, C). Analysis of ZnT-4 showed a modest but statistically significant ($P < 0.05$) increase in PCAD subjects (116.4 $\pm$ 4.0%; n = 7) compared to NC subjects (100.0 $\pm$ 4.1%; n = 7) only in CER (Figures 3.2 A, C). ZnT-6 was significantly elevated ($P < 0.05$) in HPG of PCAD subjects (157.2 $\pm$ 12.0%; n = 7) compared to NC subjects (100 $\pm$ 9.0%; n = 7) (Figures 3.3 A, B). ZnT-6 was also significantly increased ($P < 0.05$) in CER of PCAD subjects (120.3 $\pm$ 7.0%; n = 7) compared to NC subjects (100.0 $\pm$ 13.9%; n = 7) (Figures 3.3 A, C).

To localize alterations of ZnT-1 and ZnT-6 to specific neuron populations, we performed confocal microscopy and quantitative comparisons of ZnT-1 and ZnT-6 in HPG of PCAD and NC subjects. Representative confocal images of sections double labeled for MC-1 and ZnT-1 in NC and PCAD brain are shown in Figure 3.4. NC neurons showed minimal MC-1 immunoreactivity (Figure 3.4 A, green) and relatively high ZnT-1 (Figure 3.4 B, blue). In contrast, PCAD neurons (Figure 3.4D, green) showed

pronounced MC-1 but limited ZnT-1 staining (Figure 3.4 E, blue). Figures 3.4C and 3.4F show merged images. Representative confocal images of sections double labeled for MC-1 and ZnT-6 in NC and PCAD brain are shown in Figure 3.5. NC neurons with minimal MC-1 immunoreactivity (Figure 3.5 A, green) also had low levels of ZnT-6 (Figure 3.5 B, blue). Figure 3.5C shows a merged image of MC-1 and ZnT-6 immunostaining in NC HPG neurons. Figure 3.5D shows PCAD neurons strongly MC-1 positive (green) also showed pronounced ZnT-6 immunostaining (Figure 3.5 E, blue). Figure 3.5F is an MC-1/ZnT-6 merged image of PCAD neurons. Figure 3.6 shows quantitative comparisons of MC-1, ZnT-1 and ZnT-6 immunostaining in PCAD and NC HPG. MC-1 immunostaining was significantly elevated ($P < 0.05$) in PCAD (142.3 $\pm$ 15.9%; n = 5) compared to NC neurons (100.0 $\pm$ 8.1%; n = 5). In contrast, HPG sections of PCAD brain showed a significant decrease ($P < 0.05$) in ZnT-1 immunostaining (39.5 $\pm$ 7.8%; n = 5) compared to NC subjects (100.0 $\pm$ 8.7%; n = 5). Immunoreactivity of ZnT-6 was significantly ($P < 0.05$) higher in PCAD (140.4 $\pm$ 13.5%; n = 5) compared to NC neurons (100.0 $\pm$ 9.5%; n = 5). Immunostaining results were comparable to Western Blot data. Correlation analyses showed a significant ($P < 0.05$) negative correlation (r = -0.67) between MC-1 and ZnT-1 immunostaining and a significant ($P < 0.05$) positive correlation (r = 0.71) between MC-1 and ZnT-6 immunoreactivity in PCAD neurons.

Table 3.1. Subject demographic data for NC and PCAD subjects

Group	Mean ± SEM Age (y)	Sex	Mean ± SEM PMI (h)	Median Braak score
NC	86.7 ± 2.6	N = 7; 2M, 5W	2.8 ± 0.24	I
PCAD	84.6 ± 1.9	N = 7; 3M, 4W	2.9 ± 0.28	IV*

***P** < 0.05

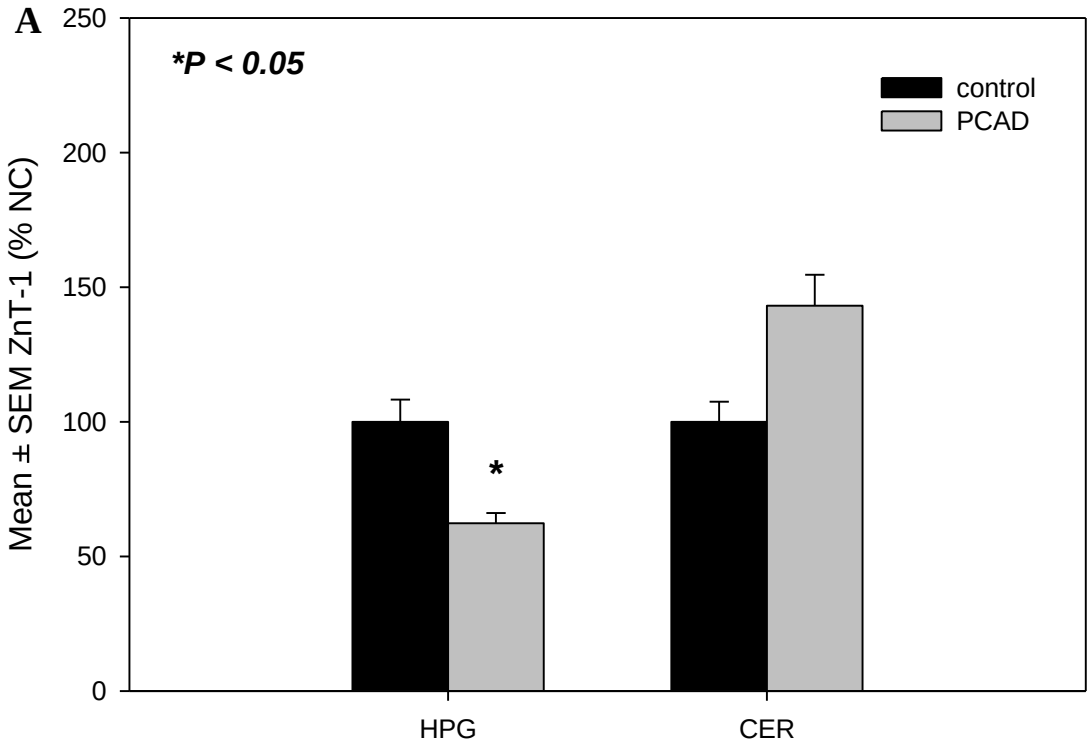

Figure 3.1.A. ZnT-1 protein levels expressed as mean ± S.E.M. (% of NC) in HPG and CER of PCAD and NC subjects. ZnT-1 was significantly decreased ($P < 0.05$) in PCAD HPG compared to age matched NC subjects.

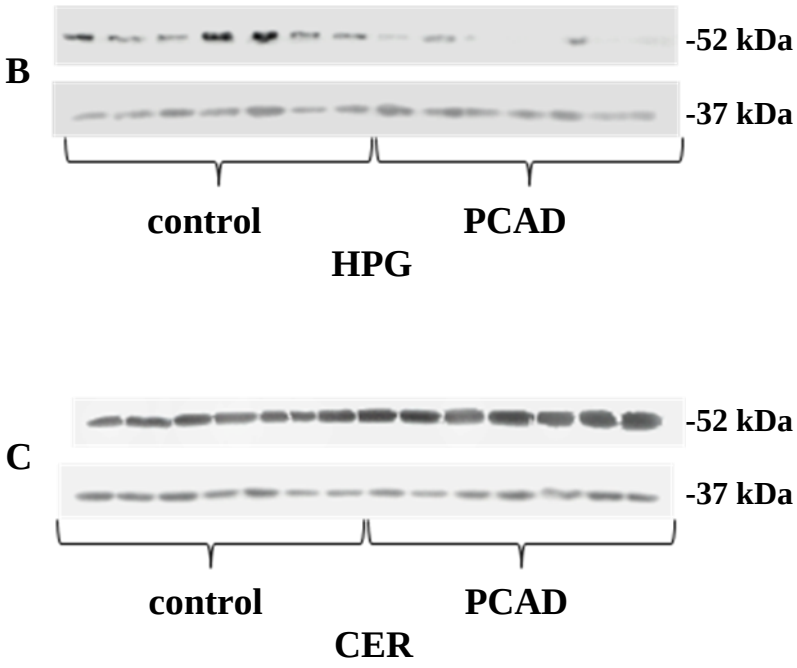

Figure 3.1.B. Representative Western blots of ZnT-1 (upper bands) and loading control (GAPDH; lower bands) in HPG of PCAD and NC subjects. **C**. Representative Western blots of ZnT-1 (upper bands) and loading control (GAPDH; lower bands) in CER of PCAD and NC subjects.

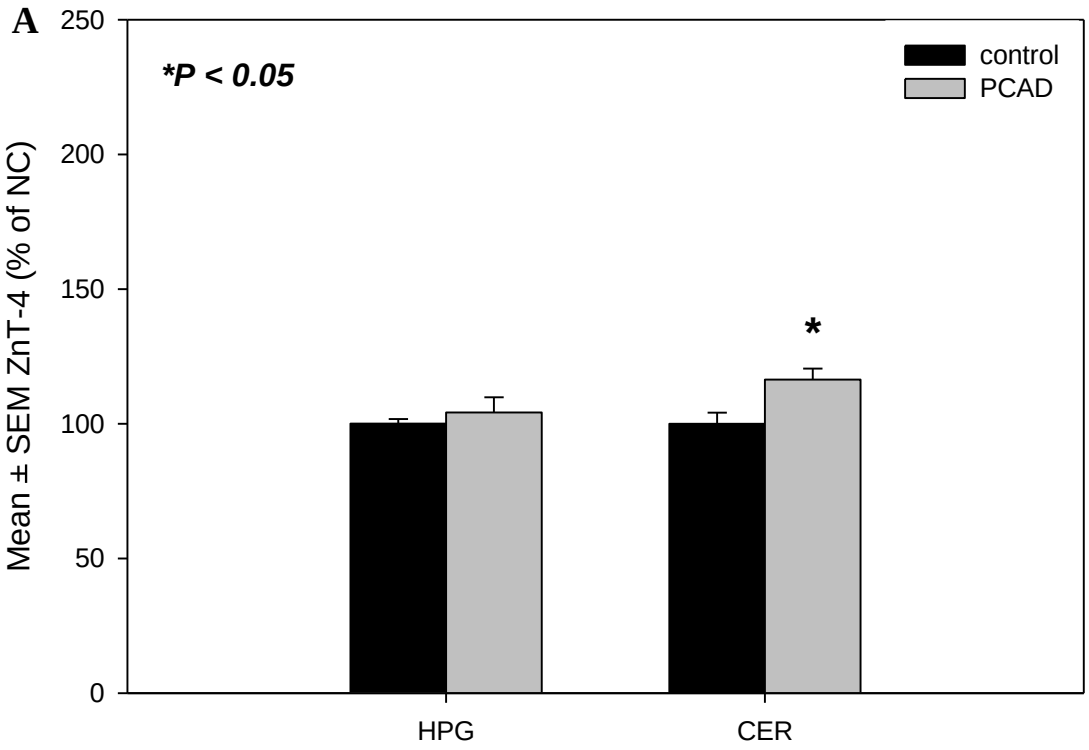

Figure 3.2.A. ZnT-4 protein levels expressed as mean ± S.E.M. (% of NC) in HPG and CER of PCAD and NC subjects. ZnT-4 was significantly increased ($P < 0.05$) in PCAD CER compared to age matched NC subjects.

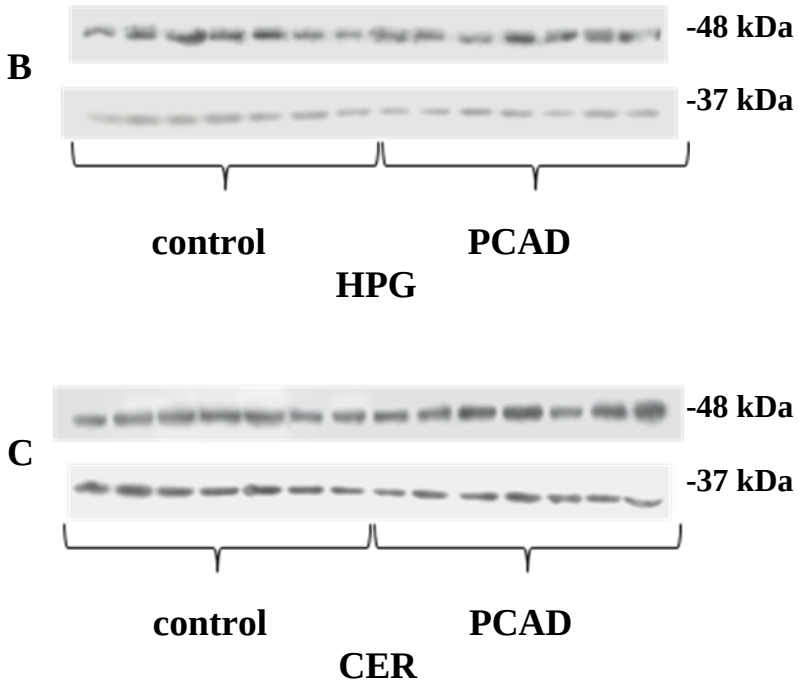

Figure 3.2.B. Representative Western blots of ZnT-4 (upper bands) and loading control (GAPDH; lower bands) in HPG of PCAD and NC subjects. **C**. Representative Western blots of ZnT-4 (upper bands) and loading control (GAPDH; lower bands) in CER of PCAD and NC subjects.

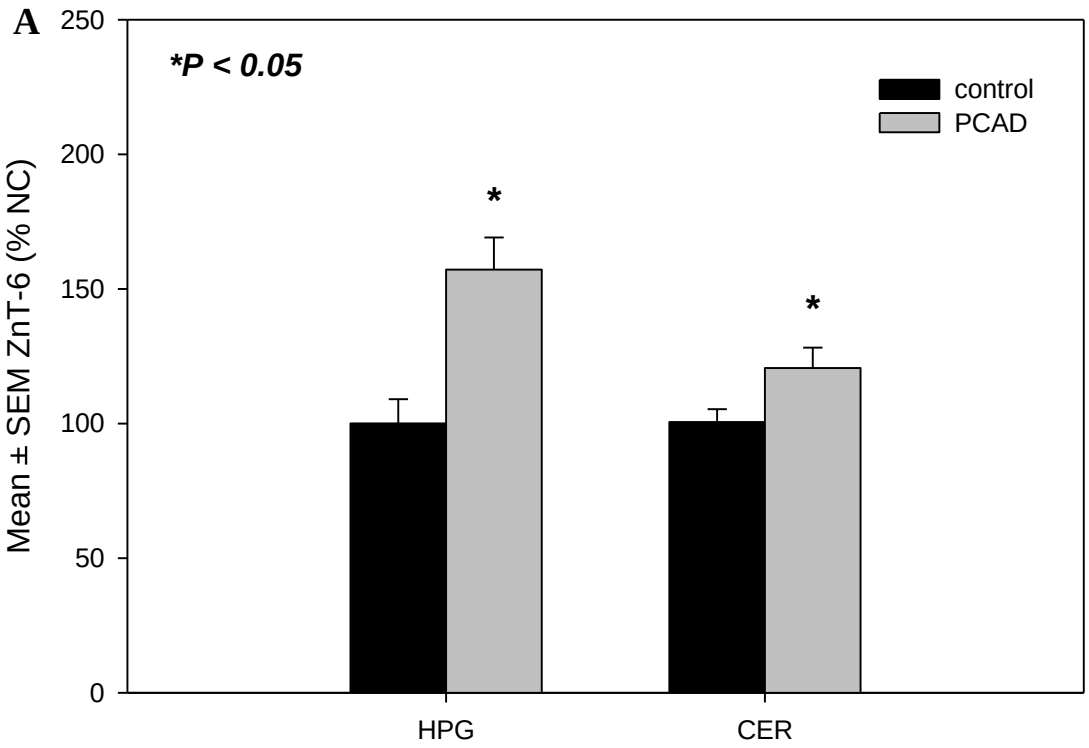

Figure 3.3.A. ZnT-6 protein levels expressed as mean ± S.E.M. (% of NC) in HPG and CER of PCAD and NC subjects. ZnT-6 was significantly increased ($P < 0.05$) in PCAD HPG and CER compared to age matched NC subjects.

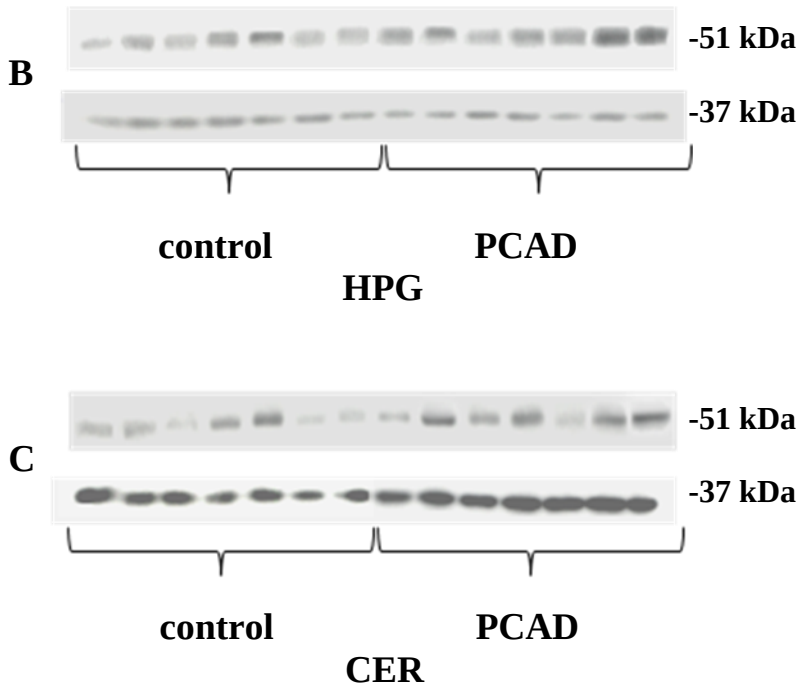

Figure 3.3.B. Representative Western blots of ZnT-6 (upper bands) and loading control (GAPDH; lower bands) in HPG of PCAD and NC subjects. **C**. Representative Western blots of ZnT-6 (upper bands) and loading control (GAPDH; lower bands) in CER of PCAD and NC subjects.

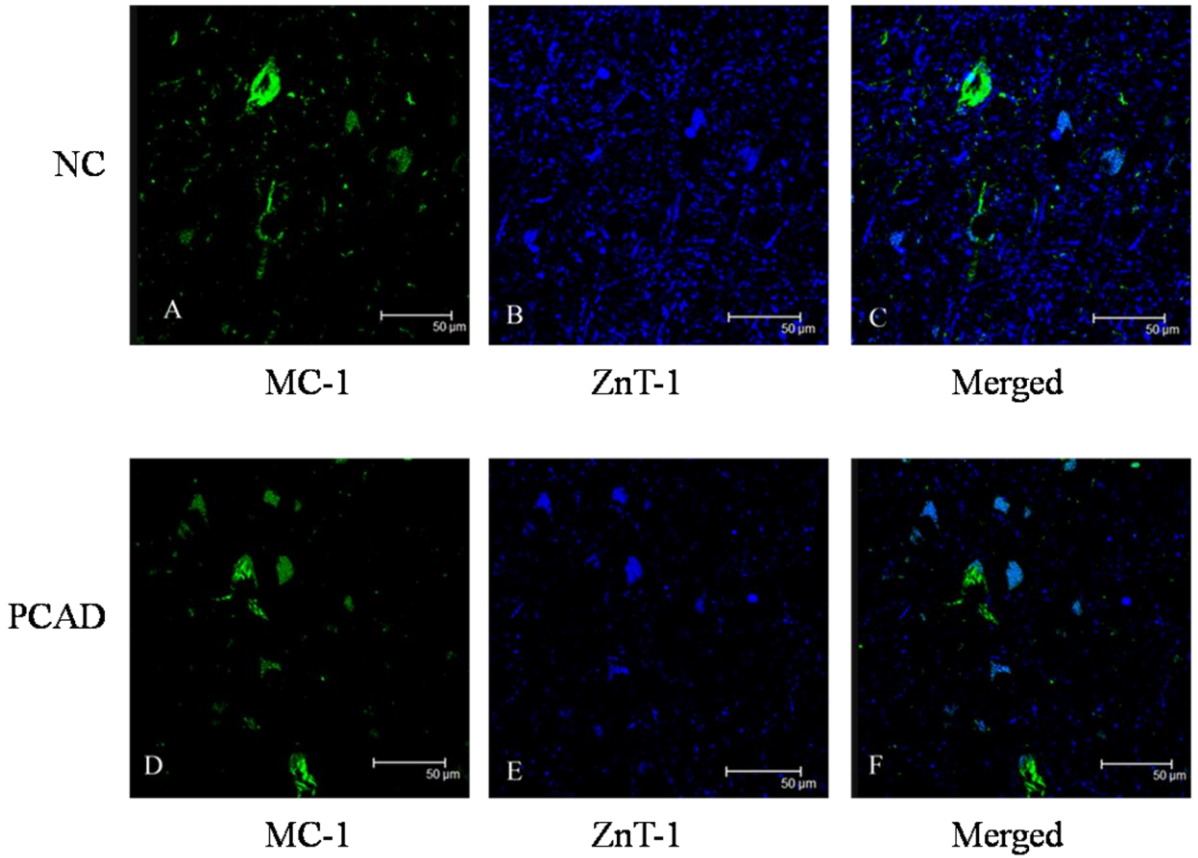

Figure 3.4. Representative confocal micrographs of NC and PCAD HPG double labeled for MC-1 (green) and ZnT-1 (blue). NC: **A.** MC-1, **B.** ZnT-1 and **C.** Merged image. PCAD: **D.** MC-1, **E.** ZnT-1 and **F.** Merged image. Note NC neurons with minimal MC-1 and relatively high ZnT-1 staining in contrast to PCAD neurons with more pronounced MC-1 and less pronounced ZnT-1 immunostaining. Scale bar = 50 µm

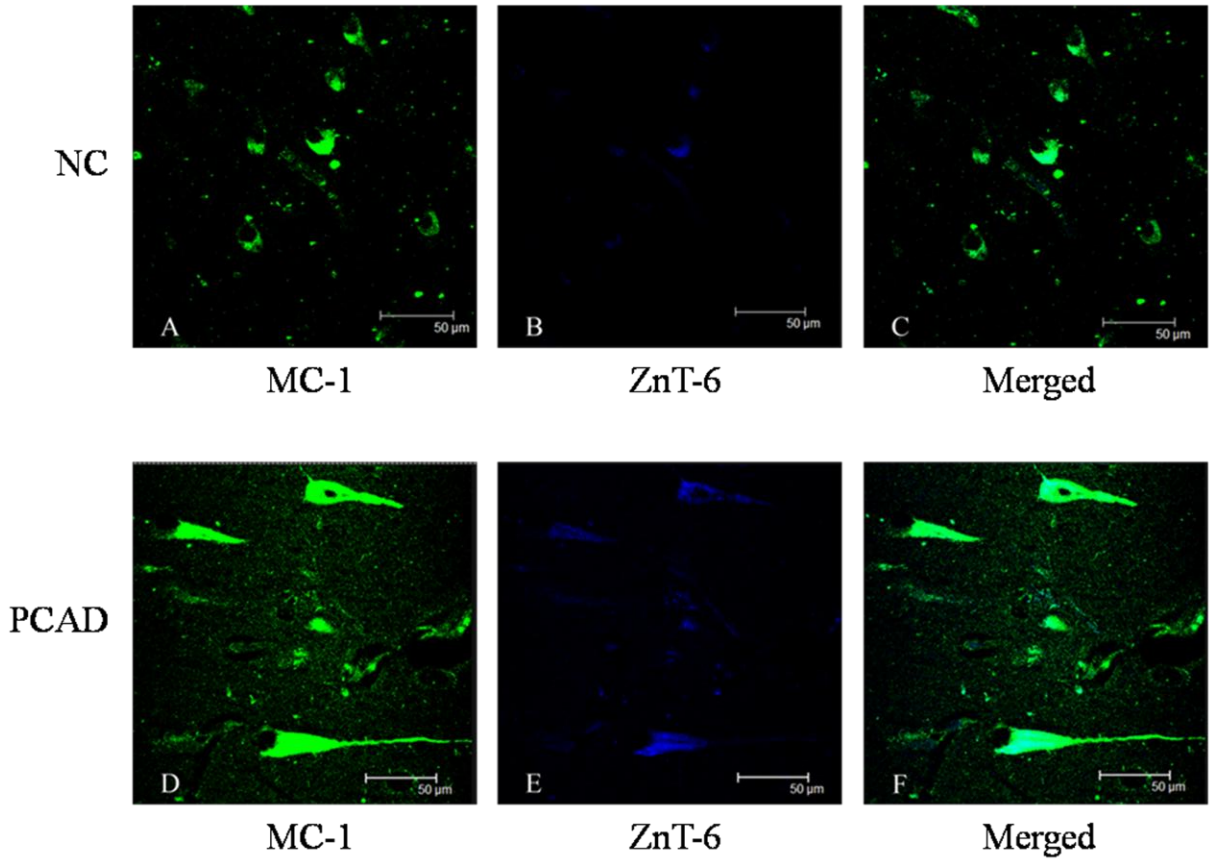

Figure 3.5. Representative confocal microscopy of neurons from NC and PCAD HPG double labeled for MC-1 (green) and ZnT-6 (blue). NC: **A.** MC-1, **B.** ZnT-6 and **C.** Merged image. PCAD: **D.** MC-1, **E.** ZnT-6 and **F.** Merged image. Note NC neurons with minimal MC-1 and ZnT-6 staining in contrast to PCAD neurons with more pronounced MC-1 and ZnT-6 immunostaining. Scale bar = 50 µm

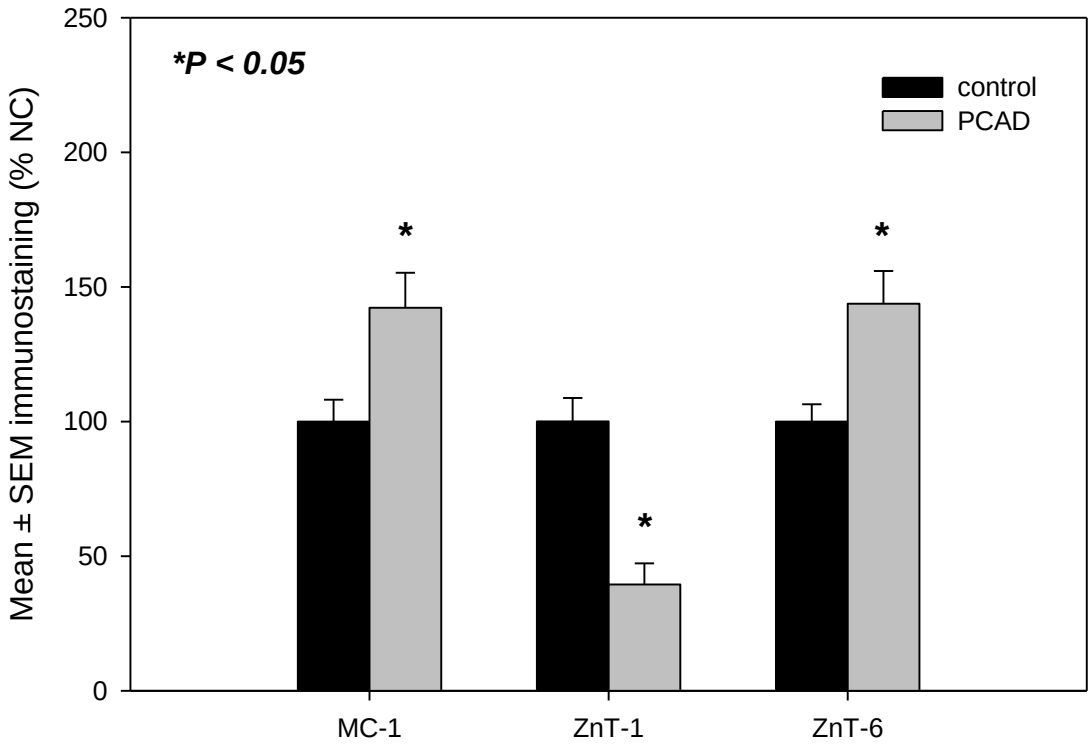

Figure 3.6. Quantitative comparisons of MC-1, ZnT-1 and ZnT-6 immunostaining in PCAD and NC HPG. MC-1 and ZnT-6 was significantly increased and ZnT-1 was significantly decreased in PCAD ($P < 0.05$) compared to age matched NC subjects.

3.2 ZnT-2 levels in the brain of subjects with preclinical Alzheimer's disease (PCAD), mild cognitive impairment (MCI), early (EAD) and late-stage Alzheimer's disease (LAD)

Subject demographic data are shown in Table 3.2. There were no significant differences between PCAD, MCI, EAD, LAD and NC subjects in age or postmortem interval (PMI). Median Braak staging scores were significantly higher in PCAD (IV), MCI (III), EAD (V) and LAD (VI) subjects compared to NC subjects (I).

Previous studies had failed to show significant levels of ZnT-2 in mouse brain so to verify the presence of ZnT-2 in human brain we carried out reverse transcriptase polymerase chain reaction (RT-PCR) amplification using primers specific for human ZnT-2. The band corresponding to the cDNA of ZnT-2 is shown on the Figure 3.7. In order to confirm that the RT-PCR product generated is the product of the *ZnT-2* gene, we extracted the cDNA for sequencing at the University of California, Davis. Sequencing results show the RT-PCR product (~ 800 base pairs) has 100% sequence homology to human ZnT-2 with a high degree of probability. ZnT-2 protein on the Western blot was visualized with a band at ~ 52 kDa. The specificity of ZnT-2 antibody was verified by pre-incubation of the antibody with blocking (immunizing) peptide that led to decreased intensity of the immunostaining band (Figure 3.8).

Figures 3.9, 3.10, 3.11 and 3.12 show ZnT-2 Western blots and quantification of immunostaining intensity levels of ZnT-2 reported as mean $\pm$ S.E.M. (% of NC) in HPG, SMTG, and CER of PCAD, MCI, EAD, LAD and age-matched NC subjects. There was a significant decrease ($P < 0.05$) of ZnT-2 in PCAD HPG (80.9 $\pm$ 5.0%; n = 5) compared to controls (100.0 $\pm$ 3.7%; n = 4). In contrast, we observed a significant elevation ($P < 0.05$) of ZnT-2 in PCAD SMTG (128.2 $\pm$ 5.8%; n = 5) compared to age-matched NC subjects (100.0 $\pm$ 10.3%; n = 4). ZnT-2 levels in CER of PCAD and NC subjects did not differ significantly (Figure 3.9). The level of ZnT-2 protein in SMTG of MCI subjects was significantly ($P < 0.05$) increased (162.9 $\pm$ 10.4%; n = 5) compared to controls (100 $\pm$ 10.8%; n = 4) (Figure 3.10). ZnT-2 levels in HPG and CER were not significantly different for MCI and NC subjects. ZnT-2 was significantly elevated ($p < 0.05$) in HPG of EAD subjects (132.4 $\pm$ 8.1%; n = 5) compared to controls (100 $\pm$ 3.0%; n = 4) (Figure

3.11). In the LAD brains ZnT-2 was significantly increased ($P < 0.05$) in HPG and SMTG (135.8 $\pm$ 10.5%; n = 5) compared to age-matched NC subjects (100.0 $\pm$ 4.8%; n = 4) (Figure 3.12). However, in CER of LAD ZnT-2 was significantly increased (77.3 $\pm$ 6.3%; n = 5) compared to NC subjects (100.0 $\pm$ 9.1%; n = 4).

Table 3.2. Subject demographic data for NC, PCAD, MCI, EAD and LAD subjects.

Group	Mean ± SEM Age (y)	Sex	Mean ± SEM PMI (h)	Median Braak score
NC	83.8 ± 3.4	N = 4; 2M, 2W	2.6 ± 0.4	I
PCAD	84.6 ± 1.9	N = 5; 3M, 2W	2.7 ± 0.3	IV*
MCI	88.2 ± 1.9	N = 5; 2M, 3W	2.5 ± 0.1	III*
EAD	89.2 ± 2.1	N = 5; 2M, 3W	3.0± 0.3	V*
LAD	81.3 ± 3.1	N = 6; 4M, 2W	2.8 ± 0.3	VI*

P < 0.05

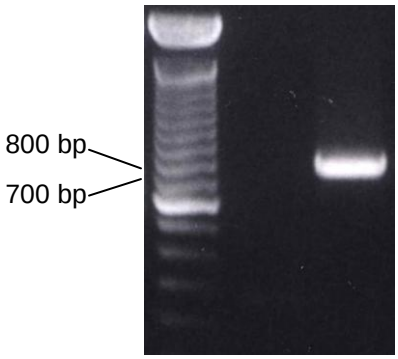

Figure 3.7. RT-PCR amplification of ZnT-2 RNA from human brain. Total RNA was isolated from representative brain specimen and subjected to RT-PCR. The RT-PCR product was run on 1.2% agarose gel and visualized with ethidium bromide. A100 bp ladder is shown in the first lane.

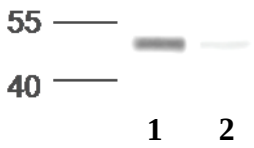

Figure.3.8. Specificity of the ZnT-2 antibody was verified using blocking peptide. Lane 2 shows inhibition of ZnT-2 immunoreactivity by blocking the antibody with immunizing peptide.

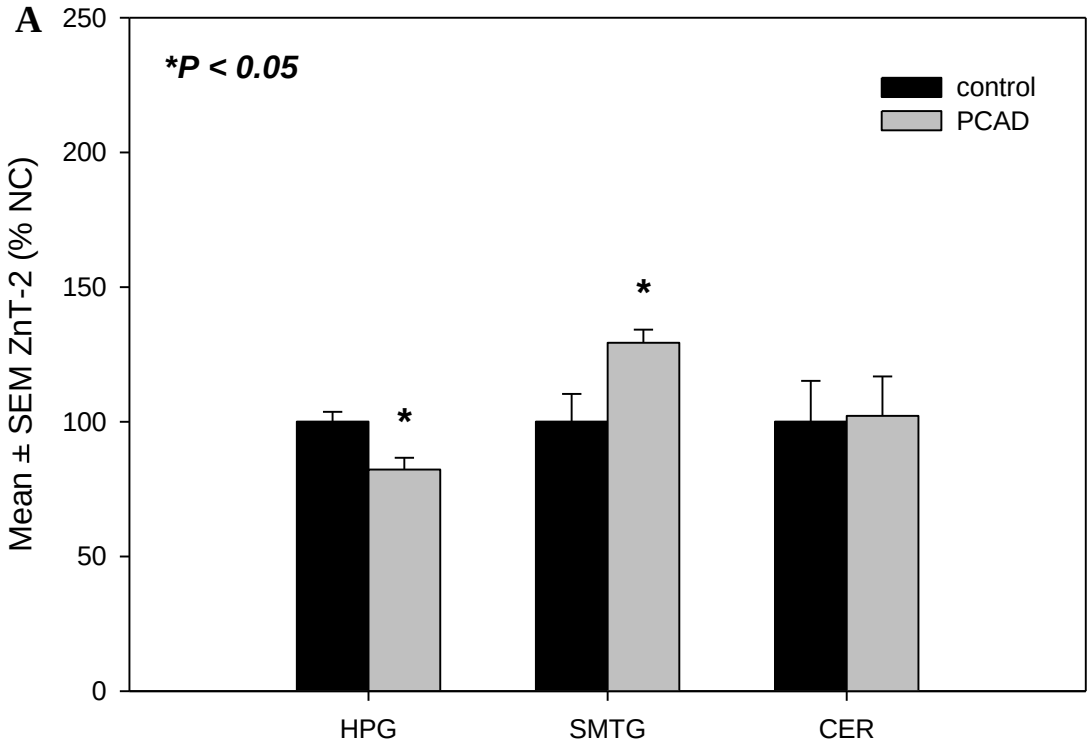

Figure 3.9.A. ZnT-2 protein levels expressed as mean ± S.E.M. (% of NC) in HPG, SMTG, and CER of PCAD and NC subjects. ZnT-2 was significantly decreased ($P < 0.05$) in PCAD HPG and significantly increased ($P < 0.05$) in PCAD SMTG compared to age matched NC subjects.

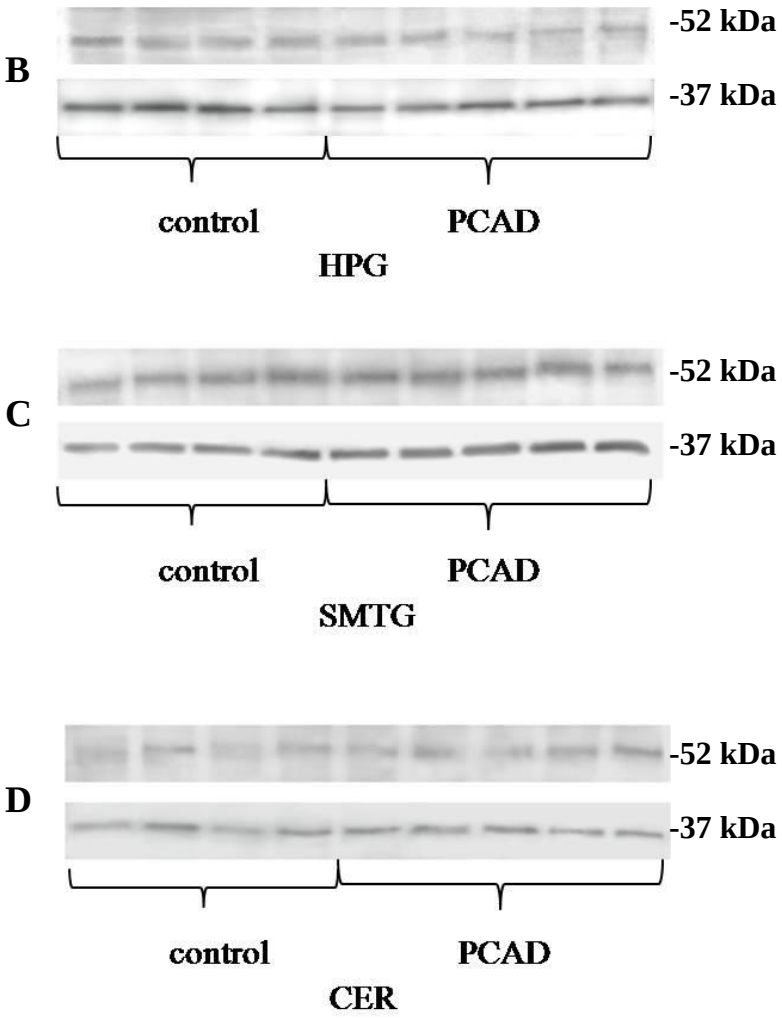

Figure 3.9.B. Representative Western blots of ZnT-2 (upper bands) and loading control (GAPDH; lower bands) in HPG of PCAD and NC subjects. **C**. Representative Western blots of ZnT-2 (upper bands) and loading control (GAPDH; lower bands) in SMTG of PCAD and NC subjects. **D.** Representative Western blots of ZnT-2 (upper bands) and loading control (GAPDH; lower bands) in CER of PCAD and NC subjects.

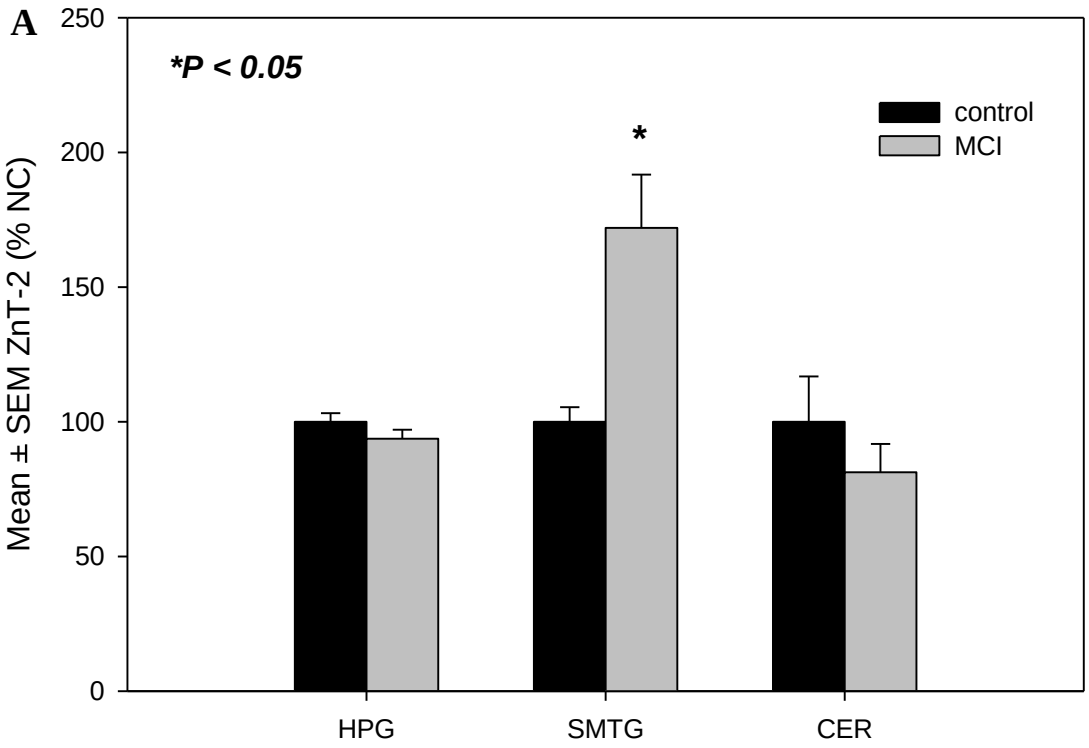

Figure 3.10.A. ZnT-2 protein levels expressed as mean ± S.E.M. (% of NC) in HPG, SMTG, and CER of MCI and NC subjects. ZnT-2 was significantly increased ($P < 0.05$) in MCI SMTG compared to age matched NC subjects.

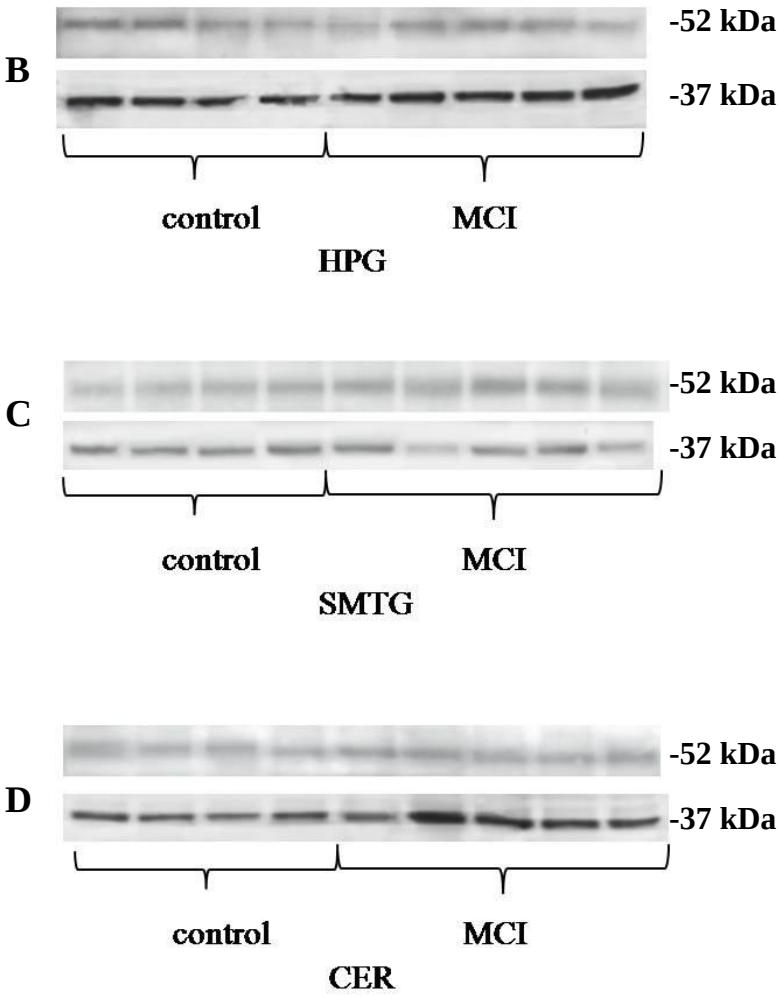

Figure 3.10.B. Representative Western blots of ZnT-2 (upper bands) and loading control (GAPDH; lower bands) in HPG of MCI and NC subjects. **C**. Representative Western blots of ZnT-2 (upper bands) and loading control (GAPDH; lower bands) in SMTG of MCI and NC subjects. **D.** Representative Western blots of ZnT-2 (upper bands) and loading control (GAPDH; lower bands) in CER of MCI and NC subjects.

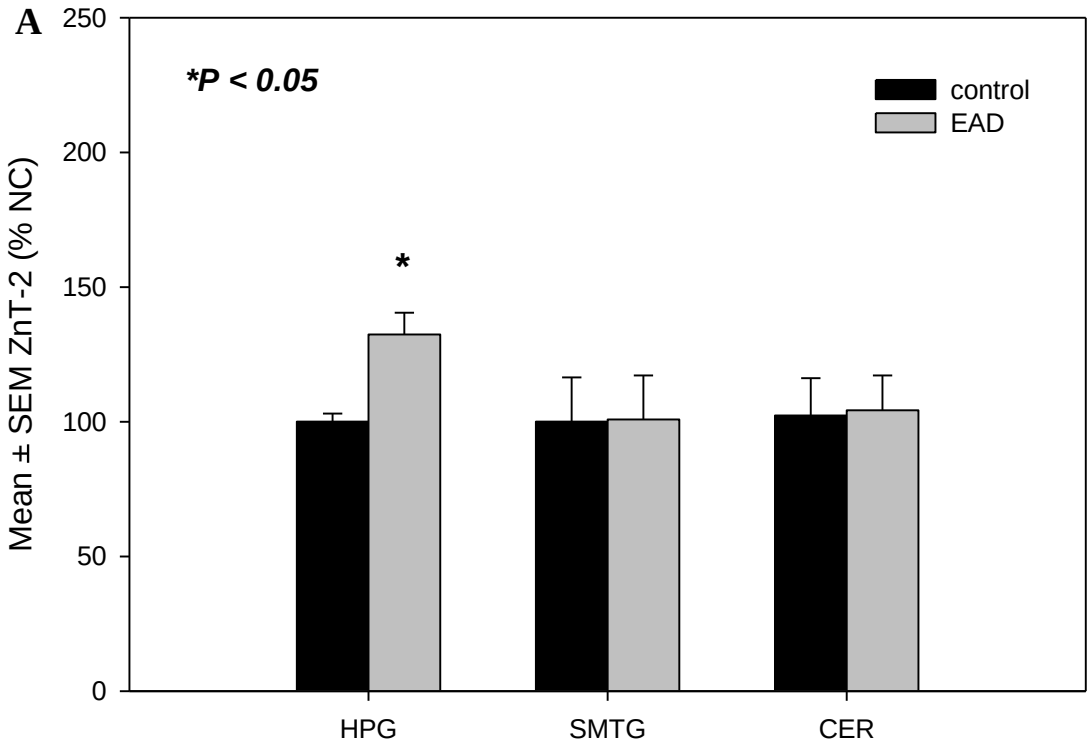

Figure 3.11.A. ZnT-2 protein levels expressed as mean ± S.E.M. (% of NC) in HPG, SMTG, and CER of EAD and NC subjects. ZnT-2 was significantly increased ($P < 0.05$) in EAD HPG compared to age matched NC subjects.

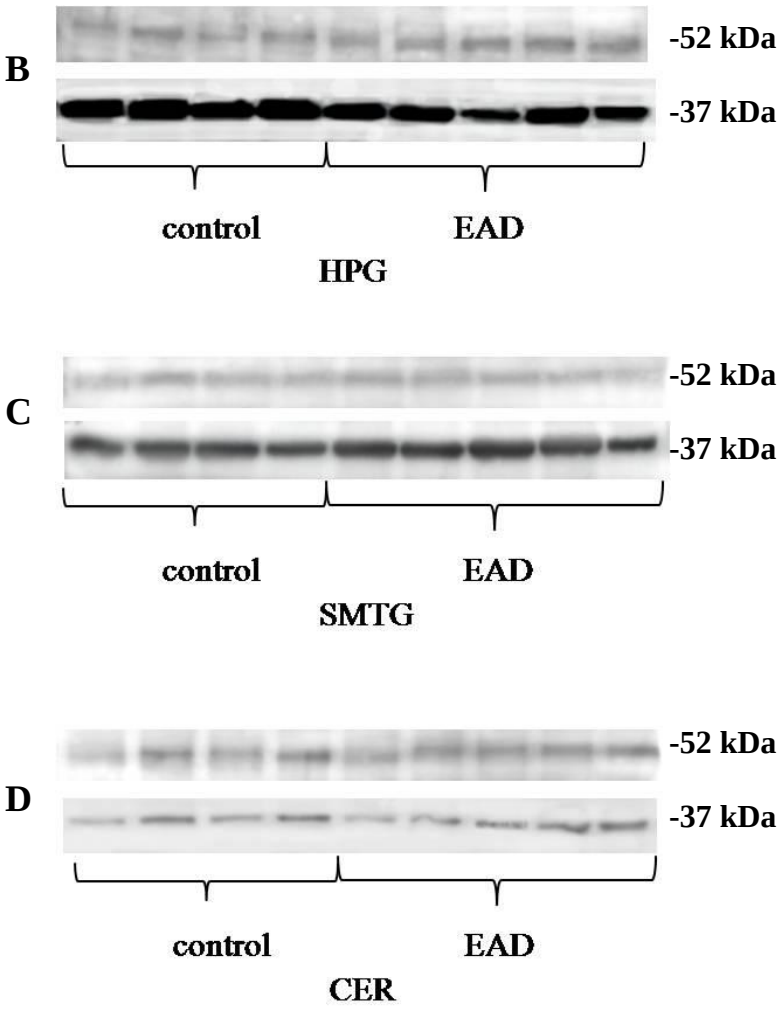

Figure 3.11.B. Representative Western blots of ZnT-1 (upper bands) and loading control (GAPDH; lower bands) in HPG of EAD and NC subjects. **C**. Representative Western blots of ZnT-2 (upper bands) and loading control (GAPDH; lower bands) in SMTG of EAD and NC subjects. **D.** Representative Western blots of ZnT-2 (upper bands) and loading control (GAPDH; lower bands) in CER of EAD and NC subjects.

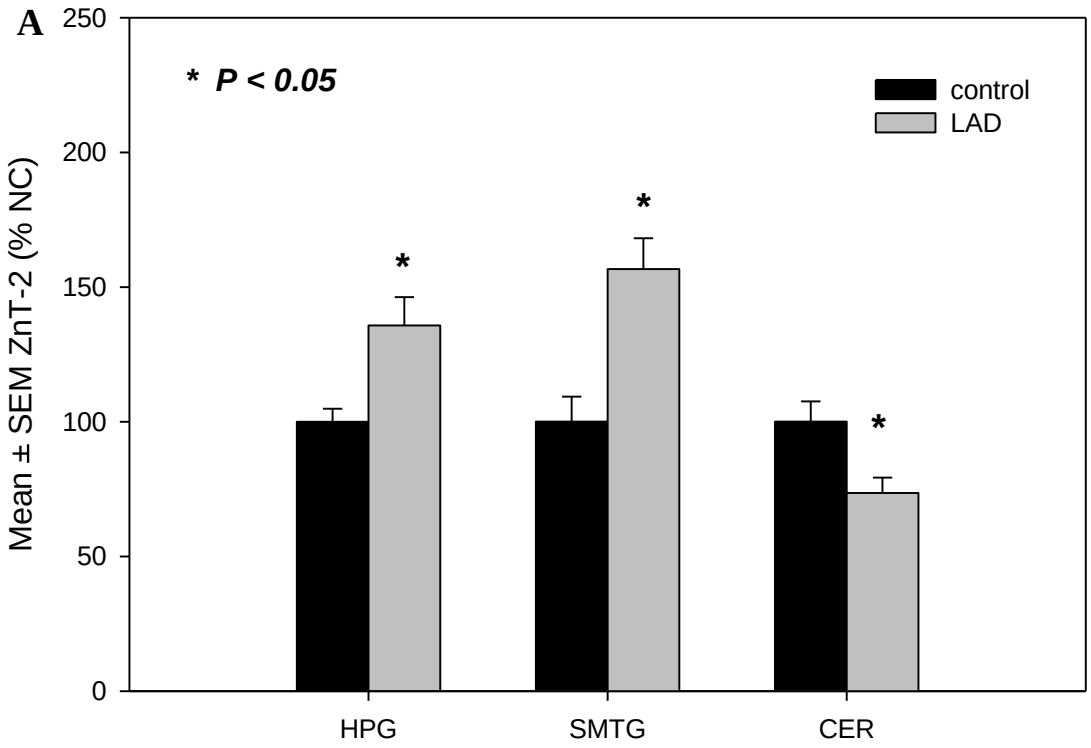

Figure 3.12.A. ZnT-2 protein levels expressed as mean ± S.E.M. (% of NC) in HPG, SMTG, and CER of LAD and NC subjects. ZnT-2 was significantly increased ($P < 0.05$) in LAD HPG and SMTG compared to age matched NC subjects. ZnT-2 was significantly decreased ($P < 0.05$) in LAD CER compared to age matched NC subjects.

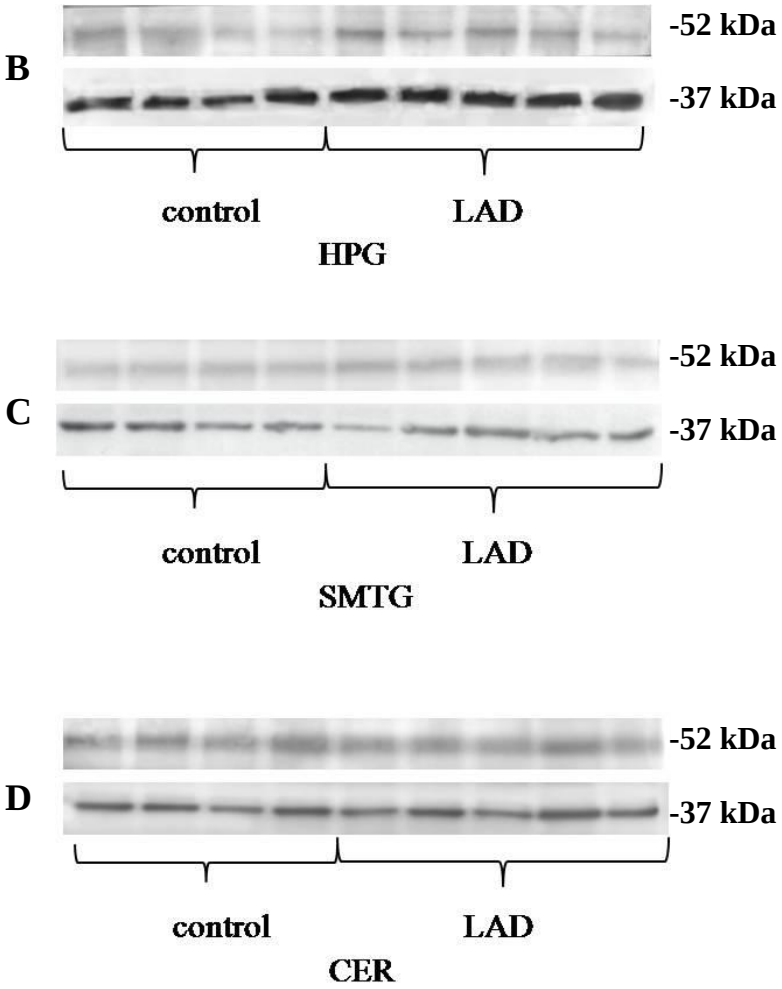

Figure 3.12.B. Representative Western blots of ZnT-1 (upper bands) and loading control (GAPDH; lower bands) in HPG of LAD and NC subjects. **C**. Representative Western blots of ZnT-2 (upper bands) and loading control (GAPDH; lower bands) in SMTG of LAD and NC subjects. **D.** Representative Western blots of ZnT-2 (upper bands) and loading control (GAPDH; lower bands) in CER of LAD and NC subjects.

3.3 A role for ZnT-1, ZnT-2, ZnT-4 and ZnT-6 in amyloid beta (Aβ) peptide processing

3.3.1 Temporal profile of ZnT-1 protein levels in H4 neuroglioma cells following Zn exposure

Changes in ZnT levels during a 16 hour Zn treatment showed considerable variation (Figures 3.13, 3.17, 3.21, 3.25). ZnT-1 protein levels rose significantly ($P < 0.05$) 0.5 h after Zn exposure (123.1 ± 0.7%) and remained elevated at 2 h (125.2 ± 2.2%), 4 h (125.1 ± 6.5%), and 8 h (119.8 ± 6.5%) but declined to (104.0 ± 5.5%) levels at 16 h, that were not significantly different from controls (100.0 ± 7.3%) (Figure 3.13).

3.3.2 siRNA mediated reduction of ZnT-1 protein levels

We used a sequence specific ZnT-1 siRNA to reduce its expression in H4-APP cells, and quantified protein levels using Western blot analysis. Figure 3.14 shows ZnT-1 protein levels expressed as mean ± S.E.M. (% of control). Controls (C+Zn) are H4-APP cells that were not treated with siRNA, but plated at the same time and subjected to the same Zn treatment as siRNA treated cells (C) (50 μM $ZnSO_4$ for 8 hours). Note that controls with Zn treatment (C+Zn) had significantly ($P < 0.05$) increased ZnT-1 levels (119.3 ± 3.9%) compared to those without Zn treatment (C) (100.0 ± 7.3%). That corresponds well with the temporal profile of ZnT-1 at 8 hours of Zn treatment (Figure 3.13). We probed the effectiveness of ZnT-1 siRNAs over a 24 hour period of Zn exposure and chose an 8 hour treatment for ZnT-1 studies because that was the time when ZnT-1 levels were high and the siRNA was the most effective in reducing ZnT-1 levels. Gene specific siRNA sequence #34 led to a significant ($P < 0.05$) decrease in ZnT-1 protein levels (70.1 ± 5.3%) compared to controls (100.0 ± 7.3%). Treatment of cells with a non gene-specific siRNA sequence, negative control (N+Zn), did not result in significant ($P < 0.05$) changes in ZnT-1 levels compared to C+Zn. At the same time, there was no significant ($P < 0.05$) change in the levels of loading control protein, GAPDH, between transfected and control cells. These results demonstrate that siRNA #34 was effective in selectively attenuating levels of ZnT-1 in H4-APP cells.

To determine if ZnT-1 expression plays a role in Aβ production, we quantified total Aβ ($A\beta_{1-40}$ and $A\beta_{1-42}$) in medium from cells treated with a ZnT-1 specific siRNA #34 which was effective in reducing ZnT-1 protein levels as well as a non specific siRNA (N+Zn) (Figure 3.15). Cultures treated with Zn but no siRNA (C+Zn) showed significantly ($P < 0.05$) increased Aβ secretion into the medium (145.7 ± 5.4%) compared to C (100.0 ± 11.9%). Cultures transfected with ZnT-1 siRNA #34 followed by Zn treatment produced significantly ($P < 0.05$) more Aβ (141.9 ± 7.8%) compared to controls without Zn treatment (C), but not significantly different compared to control cultures treated with Zn (C+Zn). Cultures transfected with a negative control siRNA followed by Zn treatment (N+Zn) did not show any significant ($P < 0.05$) changes in Aβ concentrations compared to C+Zn.

To determine the effect of ZnT-1 suppression on other ZnT proteins we analyzed levels of ZnT-2, ZnT-4 and ZnT-6 in H4-APP cells treated with siRNA #34 (Figure 3.16). We found no significant changes ($P < 0.05$) in the levels of ZnT-2, ZnT-4 and ZnT-6 in cultures transfected with ZnT-1 siRNA compared to control cultures. These results suggest reducing levels of ZnT-1 has no significant ($P < 0.05$) effect on levels of other ZnT proteins in this model system.

3.3.3 Temporal profile of ZnT-2 protein levels

Unlike ZnT-1, levels of ZnT-2 were not significantly ($P < 0.05$) elevated at any time compared to controls (Figure 3.17). We were somewhat surprised by this observation because previous studies of ZnT-2 in porcine brain capillary endothelial cells (BCEC) by Bobilya et al. showed ZnT-2 was inducible by Zn treatment (Bobilya et al., 2008).

3.3.4 siRNA mediated reduction of ZnT-2 protein levels

Figure 3.18 shows ZnT-2 protein levels expressed as mean ± S.E.M. (% of C). ZnT-2 control (C) levels in cells were not significantly ($P < 0.05$) different from cultures treated with Zn (C+Zn). These results are in agreement with the temporal profile of ZnT-2 at 16 hours of Zn treatment (Figure 3.17). Treatment of cultures with gene specific siRNA #4 led to a significant ($P < 0.05$) decrease in ZnT-2 protein levels (84.2 ± 3.6%)

compared to both C (100.0 ± 2.0%) and C+Zn (106.9 ± 1.4%), following 24 h siRNA transfection. Treatment of cells with a non gene-specific siRNA sequence (N+Zn) did not result in significant ($P < 0.05$) changes in ZnT-2 levels compared to controls. Also, there was no significant ($P < 0.05$) change in levels of loading control protein, GAPDH, between transfected and control cells.

Figure 3.19 shows cells treated with siRNA #4 generated significantly ($P < 0.05$) less Aβ (103.2 ± 6.5%) compared to those of C+Zn cells (145.7 ± 5.4%). To determine the impact of diminished ZnT-2 on other ZnT proteins we analyzed levels of ZnT-1, ZnT-4 and ZnT-6 in H4-APP cells treated with ZnT-2 siRNA #4 (Figure 3.20). There were no significant ($P < 0.05$) changes in levels of ZnT-1 and ZnT-6; but a significant decrease in levels of ZnT-4 (85.3 ± 2.4%) compared to controls (100.0 ± 1.8%), suggesting a relationship between ZnT-2 and ZnT-4. To explain our observations we initially hypothesized that siRNA #4 sequence may be complementary to the transcript of *ZnT-4* gene, but comparison of amino-acid sequences revealed that siRNA #4 was only specific for *ZnT-2*, thus our hypothesis was incorrect. Probably, we need to consider other factors (i.e. altered levels of ZIPs or MTs caused by reduced ZnT-2) which may affect ZnT-4 levels.

3.3.5 Temporal profile of ZnT-4 protein levels.

Figure 3.21 shows a time course for changes in ZnT-4 protein in H4-APP cells treated with 50 μM $ZnSO_4$ for 16 hours. After a 2 h exposure, ZnT-4 protein levels were significantly ($P < 0.05$) elevated (121.5 ± 8.1%) compared to controls (100.0 ± 5.8%). However, after 4 h of Zn treatment levels of ZnT-4 (96.8 ± 5.6%) were not significantly ($P < 0.05$) different from control cells. ZnT-4 was elevated at 8 h (113.1 ± 4.0%) compared to controls, and then again dropped to control levels at 16 h (101.2 ± 6.1%).

3.3.6 siRNA mediated reduction of ZnT-4 protein levels

Figure 3.22 shows ZnT-4 protein levels expressed as mean ± S.E.M. (% of C). Controls without Zn treatment (C) had ZnT-2 levels similar to controls treated with Zn (C+Zn) and that corresponds well with the temporal profile of ZnT-4 at 16 hours of Zn treatment (Figure 3.21). ZnT-4 gene specific siRNA sequence #24 led to a significant (*P*

< 0.05) decrease in ZnT-4 levels (38.1 ± 8.7%) compared to both C (100.0 ± 5.8%) and C+Zn (93.1 ± 2.4%), following 24 h siRNA transfection. Treatment of cells with ZnT-4 non gene-specific siRNA sequence (N+Zn) did not result in significant changes in ZnT-4 levels compared to controls. No significant difference was observed in levels of loading control protein, GAPDH, between transfected and control cells.

Medium from cells treated with siRNA#24 had significantly ($P < 0.05$) decreased Aβ concentrations (108.5 ± 10.6%) compared to those treated C+Zn cells (145.7 ± 5.4%) (Figure 3.23). We analyzed levels of ZnT-1, ZnT-4 and ZnT-6 in H4-APP cells treated with siRNA #24 (Figure 3.24). As a response to reduced ZnT-4 levels there was a significant increase ($P < 0.05$) in levels of ZnT-1 (122.7 ± 3.1%) compared to controls (100.0 ± 1.6%), and ZnT-6 (141.8 ± 11.3%) compared to controls (100.0 ± 3.0%).

3.3.7 Temporal profile of ZnT-6 protein levels

Figure 3.25 shows ZnT-6 protein levels were significantly ($P < 0.05$) increased (115.1 ± 0.1%) as early as 0.5 h after Zn treatment, compared to controls (100.0 ± 7.9%). After 2 h, however, there was no significant difference between ZnT-6 levels in treated cells and controls. The maximum effect of Zn treatment on ZnT-6 protein expression was observed at 4 h (123.7 ± 5.8%). After 8 h protein levels of ZnT-6 (105.7 ± 7.6%) were not significantly different from controls. However, they were significantly ($P < 0.05$) increased after 16 h of Zn treatment (116.3 ± 4.2%) compared to controls.

3.3.8 siRNA mediated reduction of ZnT-6 protein levels

Figure 3.26 shows ZnT-6 protein levels as mean ± S.E.M. (% of C). Controls without Zn treatment (C) had significantly ($P < 0.05$) lower ZnT-6 levels (100.0 ± 7.9%) compared to C+Zn (120.9 ± 3.2%). That corresponds well with the temporal profile of ZnT-6 after 16 h of Zn treatment (Figure 3.25). ZnT-6 sequence specific siRNA #9 led to a significant ($P < 0.05$) decrease in ZnT-6 protein levels (92.2 ± 7.0%) compared to C+Zn, following 24 h siRNA transfection. Treatment of cells with ZnT-6 non gene-specific siRNA sequence (N+Zn) did not result in significant ($P < 0.05$) changes in ZnT-6 levels compared to controls. There were no significant changes in levels of the loading control protein, GAPDH, between transfected and control cells.

Media from cells treated with siRNA #9 was characterized by significantly ($P < 0.05$) decreased Aβ levels (102.4 ± 3.9%) compared to those of C+Zn cells (145.7 ± 9.3%) (Figure 3.27). We analyzed levels of ZnT-1, ZnT-2 and ZnT-4 in H4-APP cells treated with siRNA #9 (Figure 3.28) and found a significant increase ($P < 0.05$) in levels of ZnT-1 (168.4 ± 7.4%) compared to controls (100.0 ± 1.6%), and a significant increase ($P < 0.05$) in levels of ZnT-2 (131.2 ± 4.9%) compared to controls (100.0 ± 2.3%). In contrast, levels of ZnT-4 were significantly ($P < 0.05$) decreased (71.1 ± 6.4%) compared to controls (100.0 ± 1.8%).

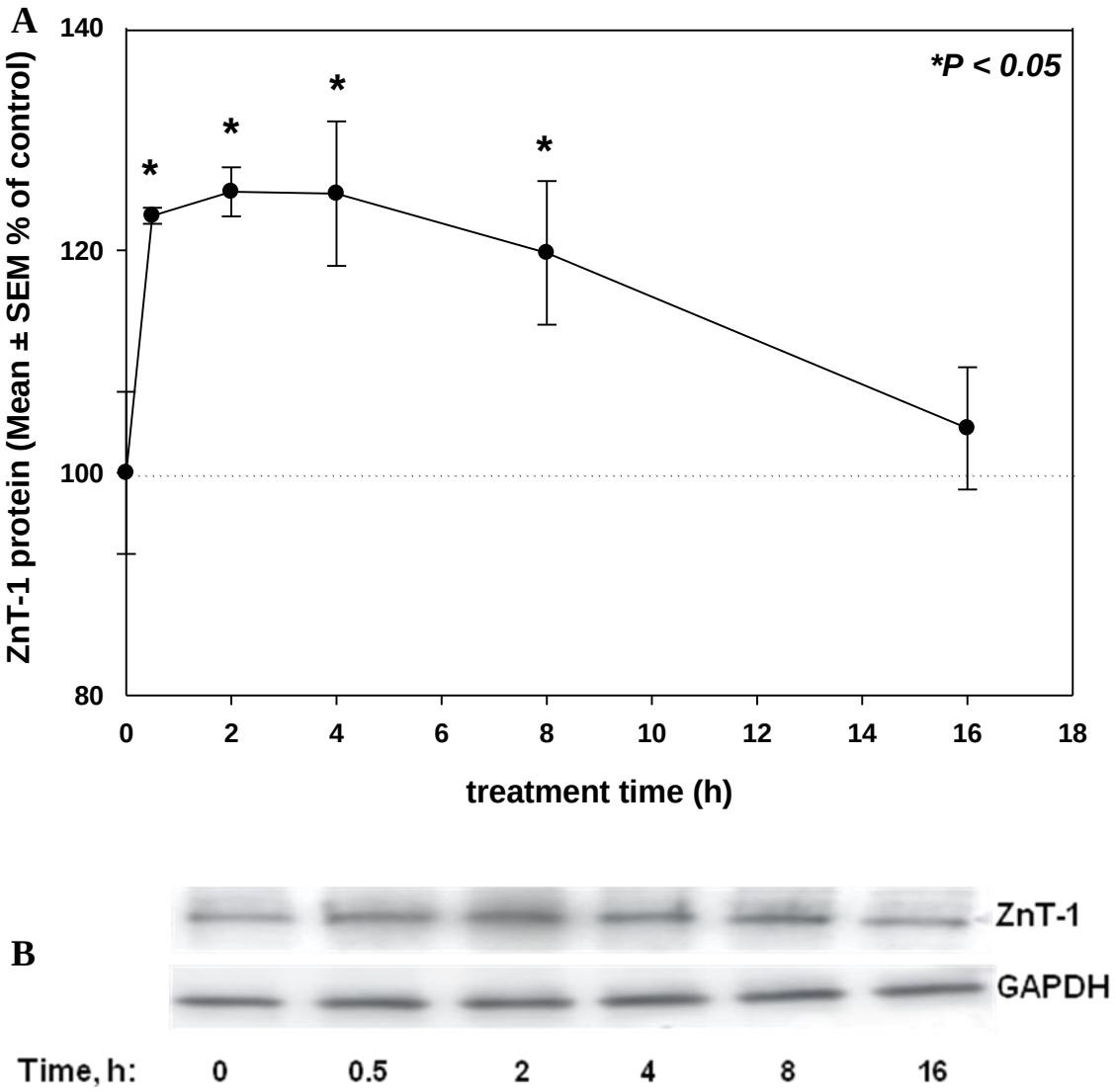

Figure 3.13. Temporal profile of ZnT-1 protein in H4-APP cells treated with 50 μM $ZnSO_4$. **A.** ZnT-4 levels are expressed as mean ± S.E.M. (% of untreated control), n = 6. **B**. Representative Western blots of ZnT-1 (upper bands) and loading control (GAPDH; lower bands) in H4-APP cells.

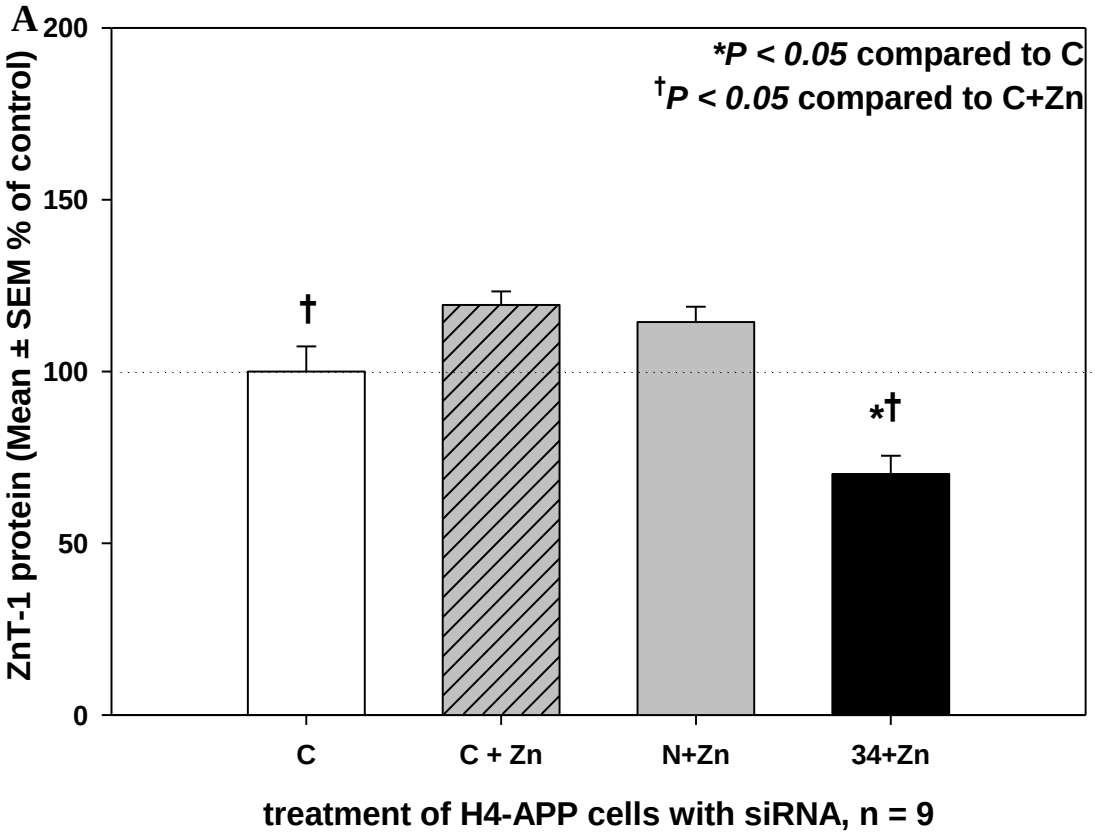

Figure 3.14. siRNA transfection was effective in reducing ZnT-1 protein levels. **A.** ZnT-1 protein levels expressed as mean ± S.E.M. (% of controls, C), n = 9. ZnT-1 sequence specific siRNA #34 led to a significant ($P < 0.05$) decrease in ZnT-1 protein levels following 24 h transfection compared to controls treated with $ZnSO_4$ but not siRNA, C+Zn. Treatment of cells with ZnT-1 non gene-specific siRNA sequence followed by $ZnSO_4$ treatment (N+Zn) did not result in significant changes in ZnT-1 levels compared to C+Zn.

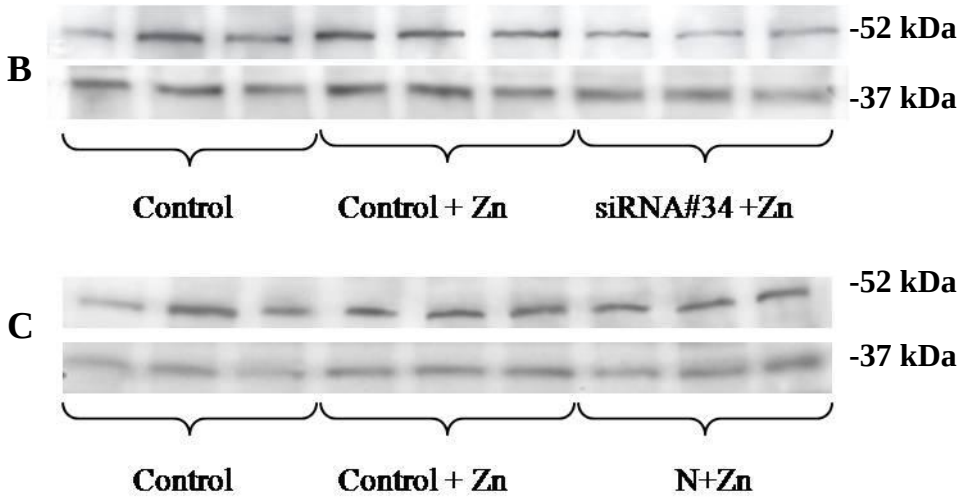

Figure 3.14.B. Representative Western blots of ZnT-1 (upper bands) and loading control (GAPDH; lower bands) for H4-APP cells treated with a gene specific siRNA #34 followed by Zn exposure. **C**. Representative Western blots of ZnT-1 (upper bands) and loading control (GAPDH; lower bands) for H4-APP cells treated with a non gene-specific siRNA sequence, negative control (N), followed by Zn exposure.

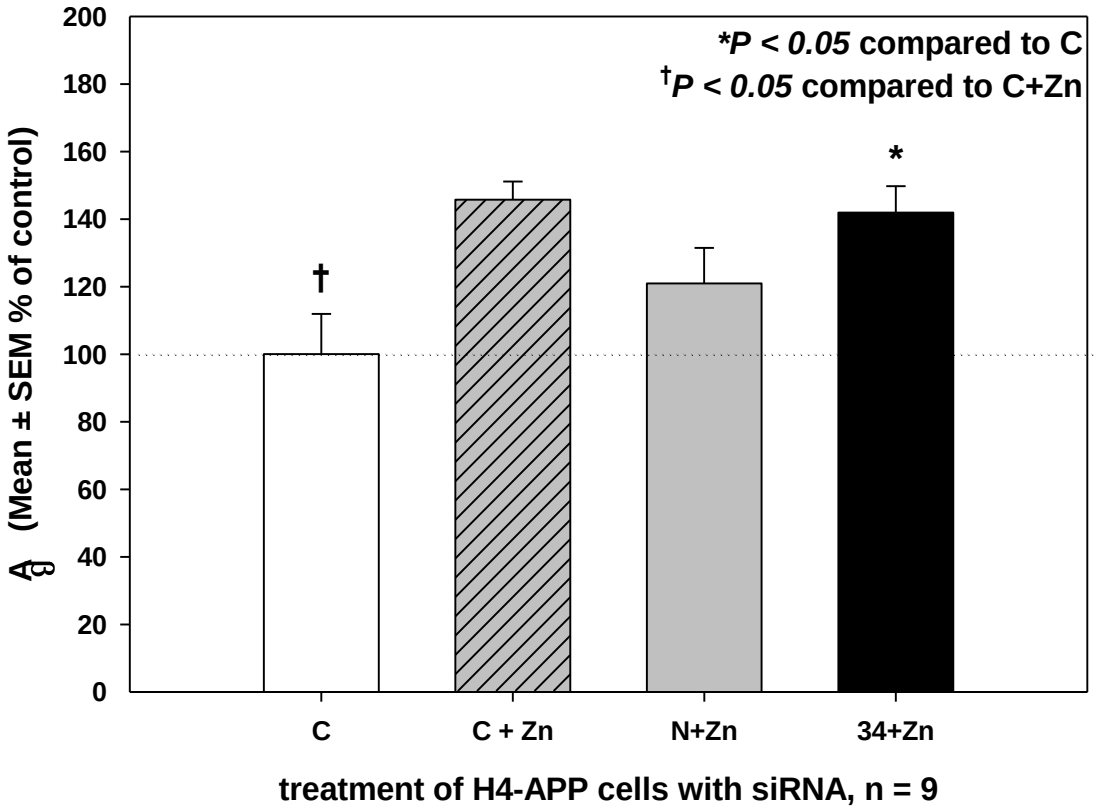

Figure 3.15. Reduction of ZnT-1 protein levels did not significantly ($P < 0.05$) affect Aβ concentrations in the cell medium. Aβ levels expressed as mean ± S.E.M. (% of controls, C), n = 9. Media from control cells treated with $ZnSO_4$ (C+Zn) had significantly ($P < 0.05$) increased Aβ levels compared to control cells without $ZnSO_4$ treatment (C). Media from cells which had suppressed levels of ZnT-1 protein (siRNA #34) showed Aβ concentrations that were significantly ($P < 0.05$) increased compared to control cells (C) but not significantly ($P < 0.05$) different from those of control cells treated with $ZnSO_4$ (C+Zn). Treatment of cells with ZnT-1 non gene-specific siRNA sequence followed by $ZnSO_4$ treatment (N+Zn) did not result in significant changes in Aβ levels compared to C+Zn.

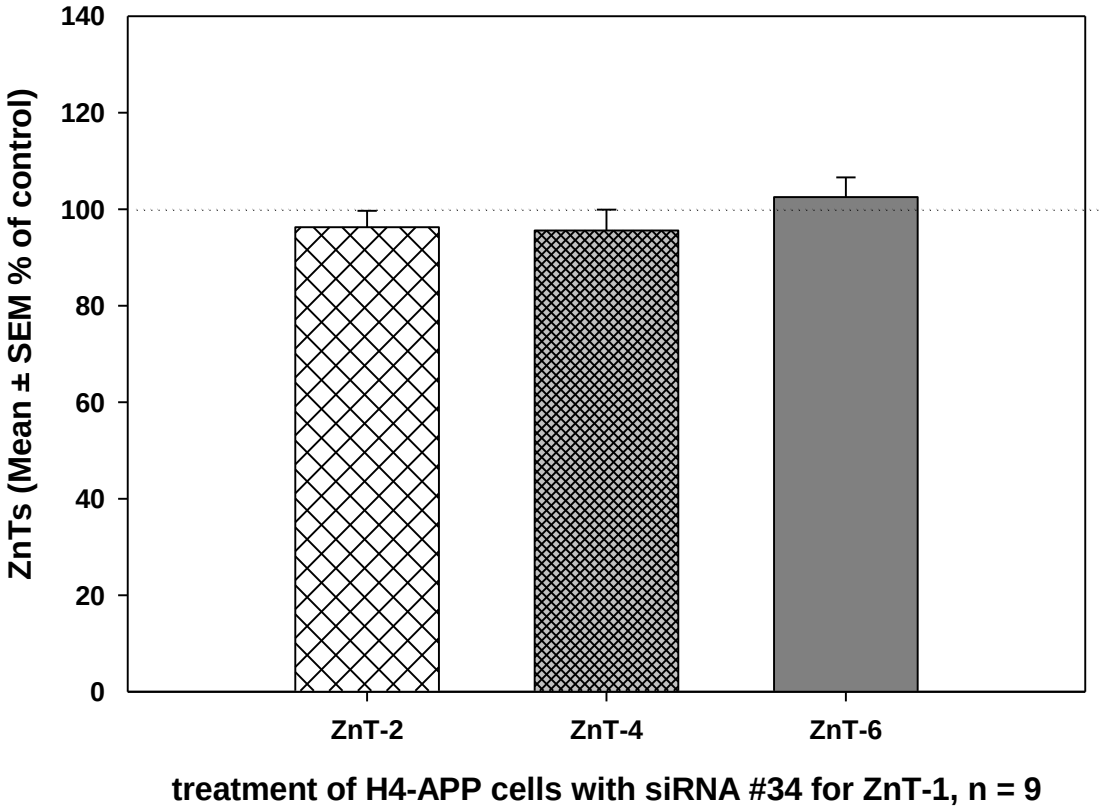

Figure 3.16. Reduction of ZnT-1 protein levels using siRNA did not significantly ($P < 0.05$) affect levels of other ZnTs. ZnT levels expressed as mean ± S.E.M. (% of controls that were not treated with siRNA, shown as C+Zn), n = 9**.**

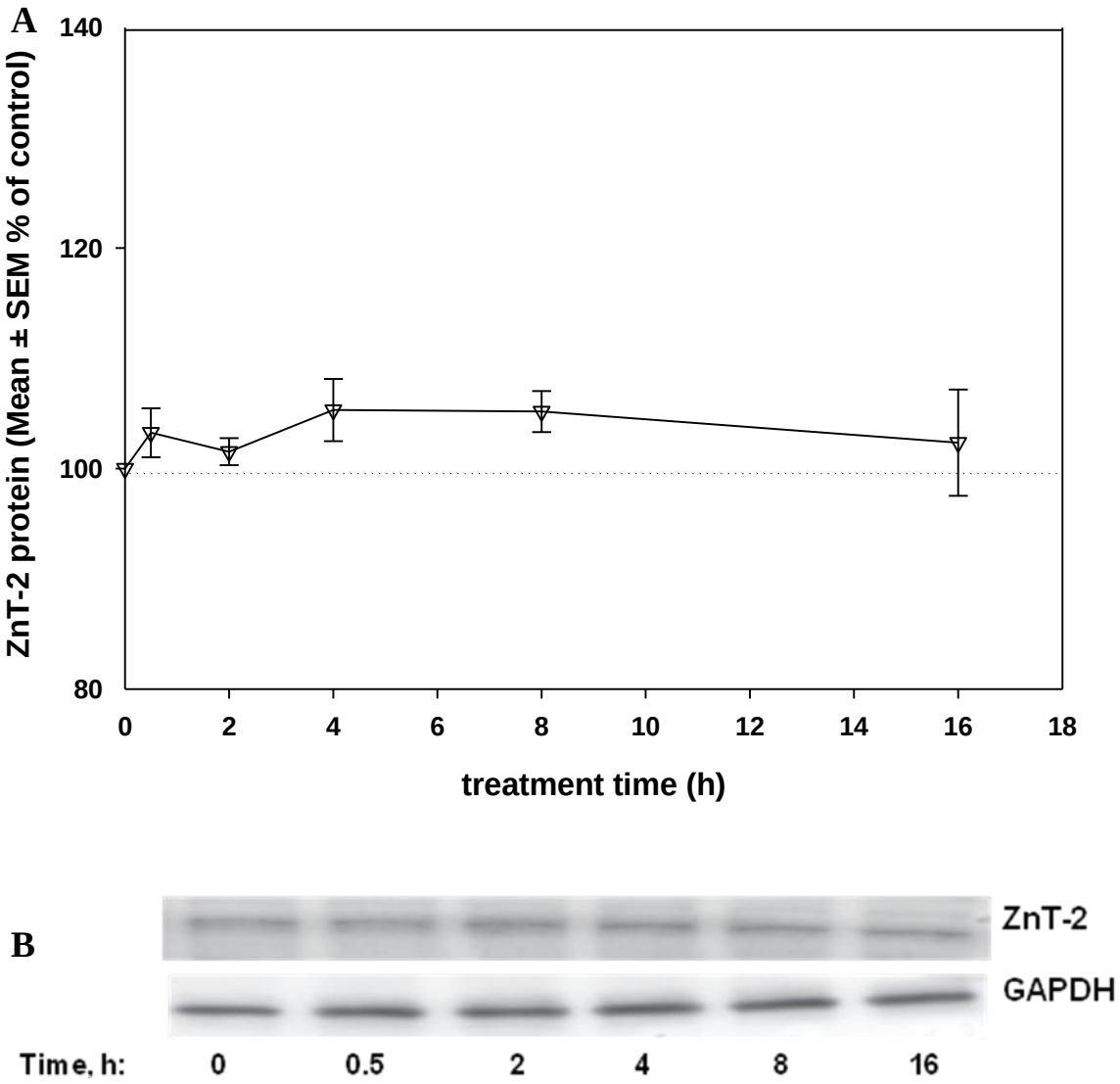

Figure 3.17. Temporal profile of ZnT-2 protein in H4-APP cells treated with 50 μM $ZnSO_4$. **A.** ZnT-2 levels are expressed as mean ± S.E.M. (% of untreated control), n = 6. **B**. Representative Western blots of ZnT-2 (upper bands) and loading control (GAPDH; lower bands) in H4-APP cells.

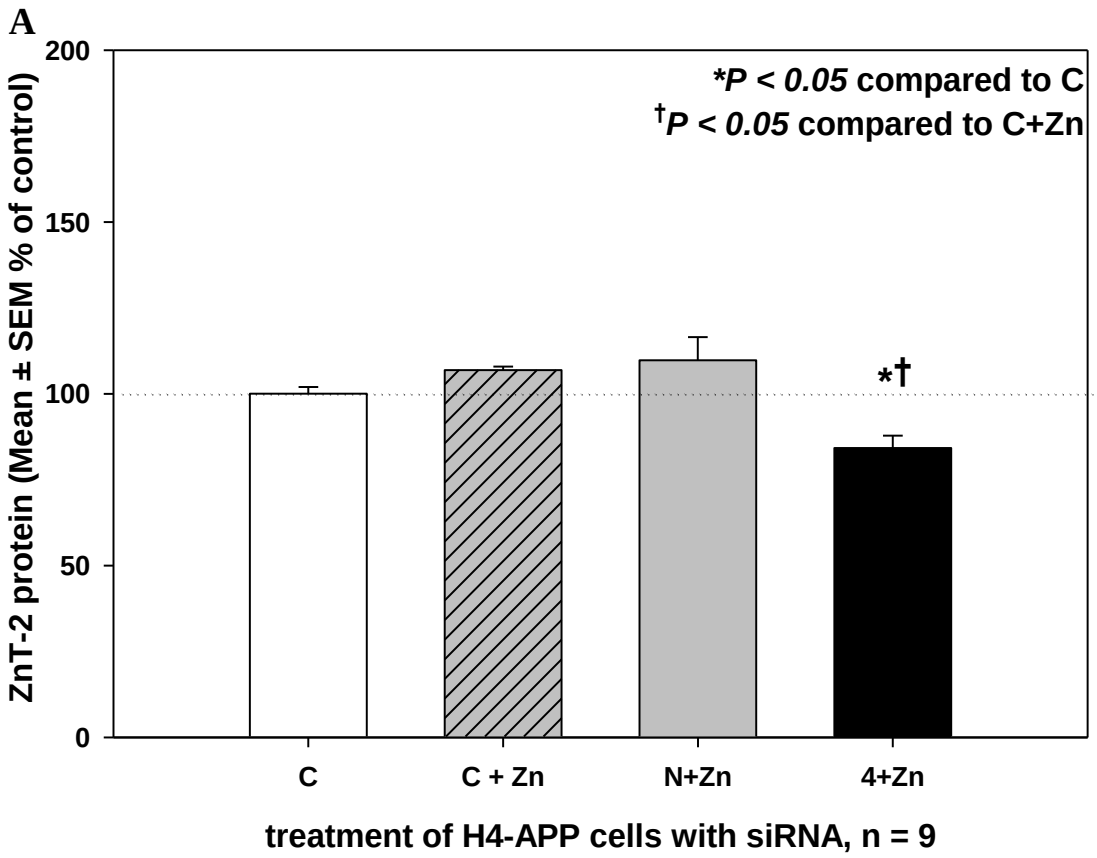

Figure 3.18. Use of a ZnT-2 siRNA was effective in reducing protein levels. **A.** ZnT-2 protein levels expressed as mean ± S.E.M. (% of controls, C), n = 9. ZnT-2 sequence specific siRNA #4 led to a significant ($P < 0.05$) decrease in ZnT-2 protein levels following 24 h transfection compared to controls treated with $ZnSO_4$ but not siRNA, C+Zn. Treatment of cells with a non gene-specific siRNA sequence followed by $ZnSO_4$ treatment (N+Zn) did not result in significant changes in ZnT-2 levels compared to C+Zn.

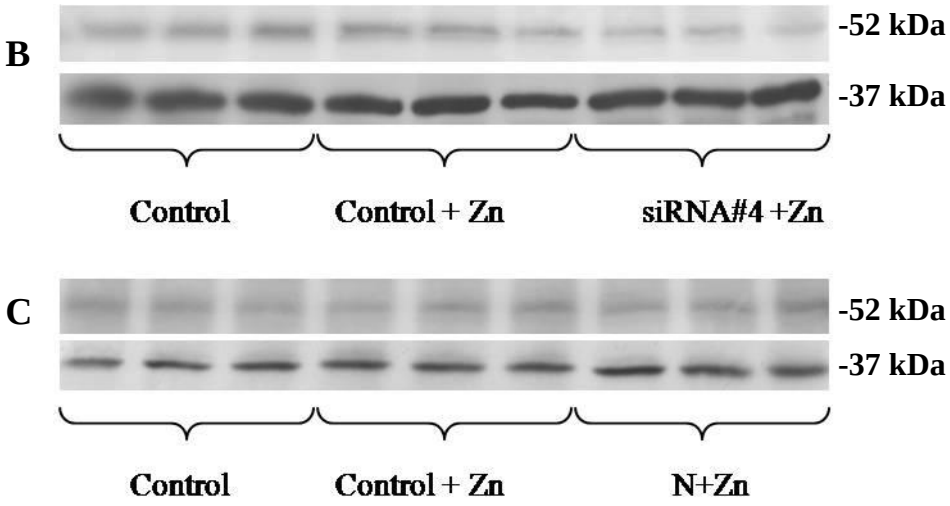

Figure 3.18.B. Representative Western blots of ZnT-2 (upper bands) and loading control (GAPDH; lower bands) for H4-APP cells treated with a gene specific siRNA #4 followed by Zn exposure. **C**. Representative Western blots of ZnT-2 (upper bands) and loading control (GAPDH; lower bands) for H4-APP cells treated with a non gene-specific siRNA sequence, negative control (N), followed by Zn exposure.

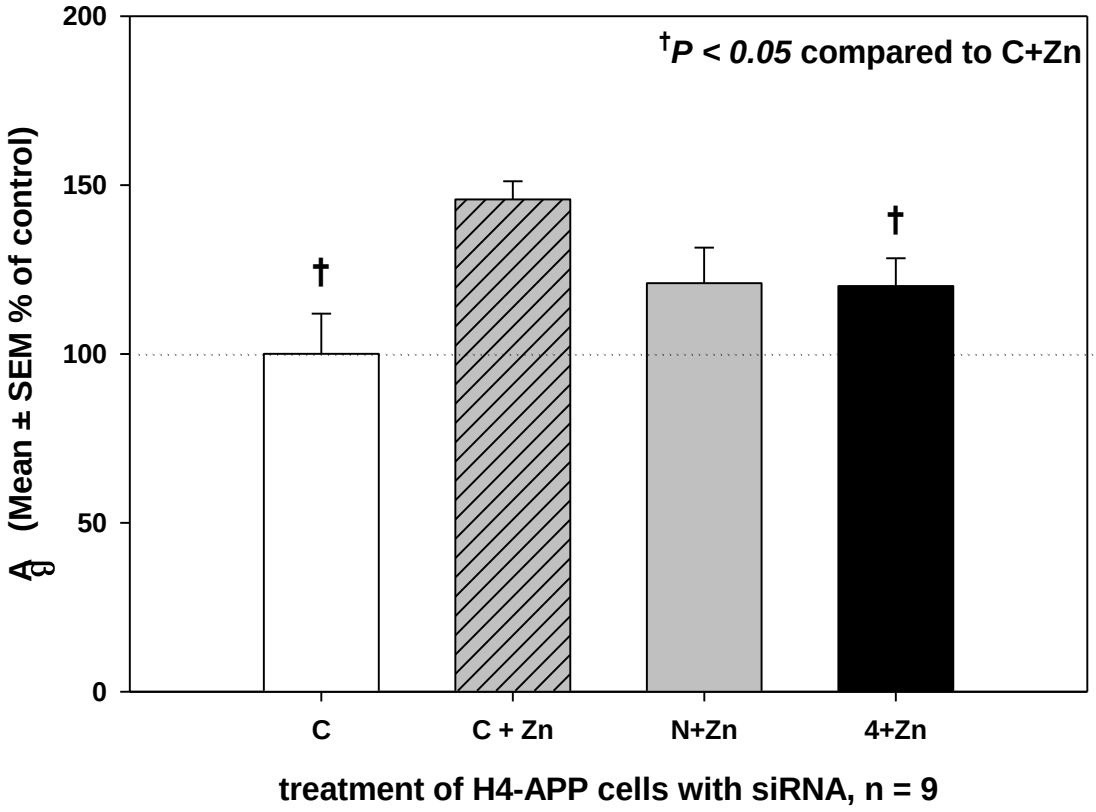

Figure 3.19. Reduction of ZnT-2 protein levels led to significantly ($P < 0.05$) decreased Aβ concentrations in the cell medium. Aβ levels expressed as mean ± S.E.M. (% of controls, C), n = 9. Medium from control cells treated with $ZnSO_4$ (C+Zn) had significantly ($P < 0.05$) increased Aβ levels compared to control cells without $ZnSO_4$ treatment (C). Medium from cells treated with siRNA #4 had significantly ($P < 0.05$) decreased Aβ concentrations compared to those of C+Zn cells. Treatment of cells with a non gene-specific siRNA sequence followed by $ZnSO_4$ treatment (N+Zn) did not result in significant ($P < 0.05$) changes in Aβ levels compared to C+Zn**.**

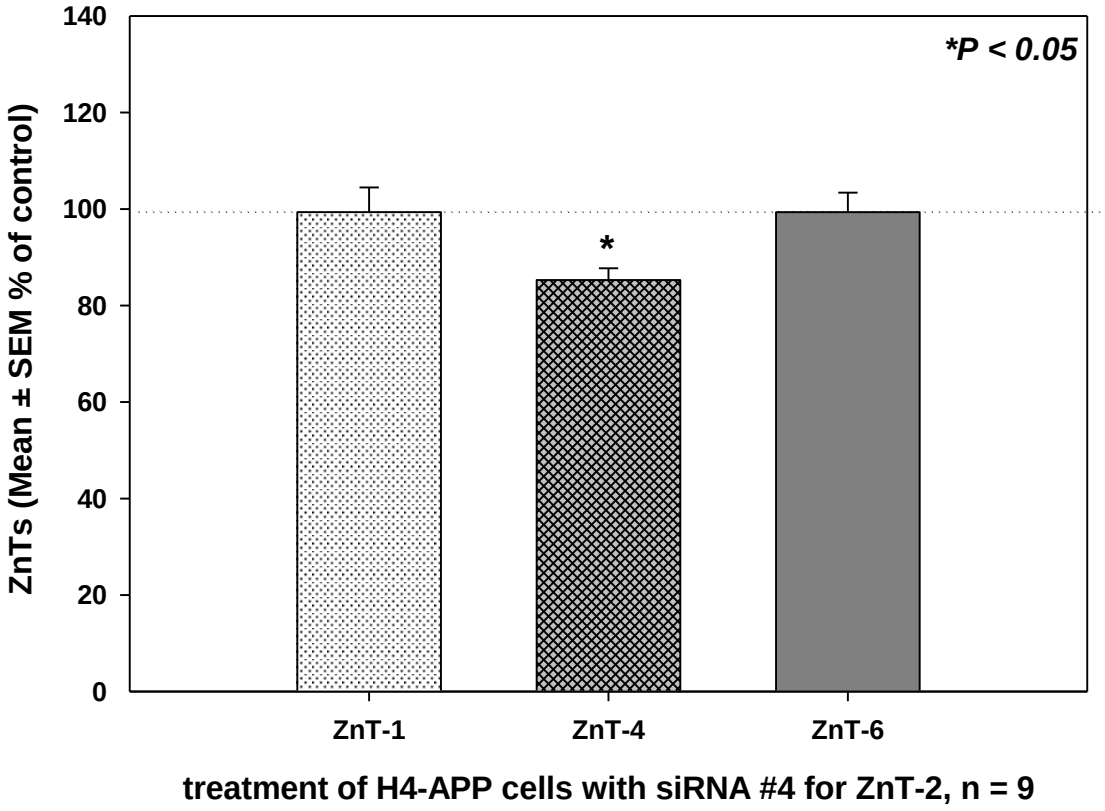

Figure 3.20. siRNA mediated reduction of ZnT-2 caused a significant ($P < 0.05$) decrease in ZnT-4 protein levels. ZnT levels expressed as mean ± S.E.M. (% of controls that were not treated with siRNA, shown as (C+Zn), n = 9.

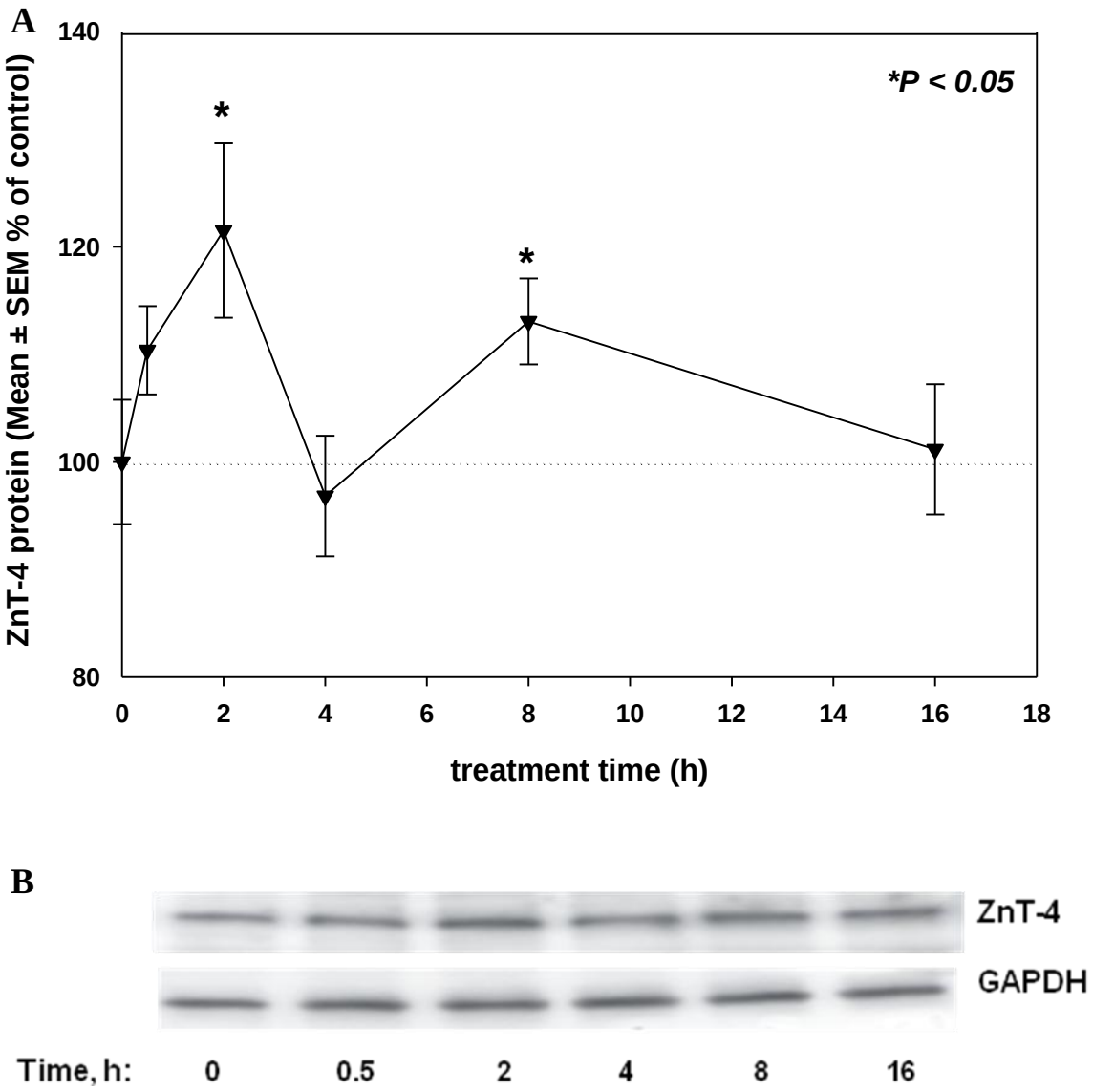

Figure 3.21. Temporal profile of ZnT-4 protein in H4-APP cells treated with 50 μM $ZnSO_4$. **A.** ZnT-4 levels are expressed as mean ± S.E.M. (% of untreated control), n = 6. **B**. Representative Western blots of ZnT-4 (upper bands) and loading control (GAPDH; lower bands) in H4-APP cells.

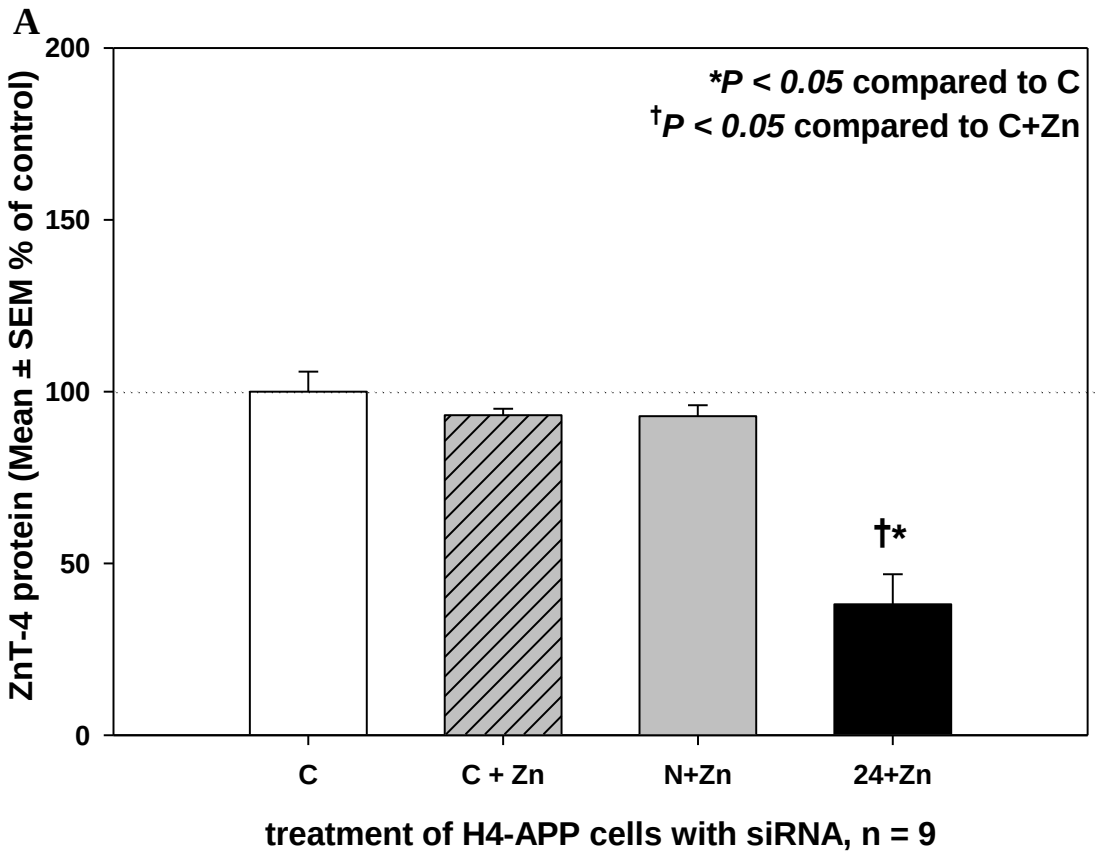

Figure 3.22. Treatment with a ZnT-4 siRNA led to decresed ZnT-4 protein levels. **A.** ZnT-4 protein levels expressed as mean ± S.E.M. (% of controls, C), n = 9. ZnT-4 sequence specific siRNA #24 led to a significant ($P < 0.05$) decrease in ZnT-4 protein levels following 24 h transfection compared to controls treated with $ZnSO_4$ but not siRNA, C+Zn. Treatment of cells with ZnT-4 non gene-specific siRNA sequence followed by $ZnSO_4$ treatment (N+Zn) did not result in significant changes in Aβ levels compared to both C and C+Zn.

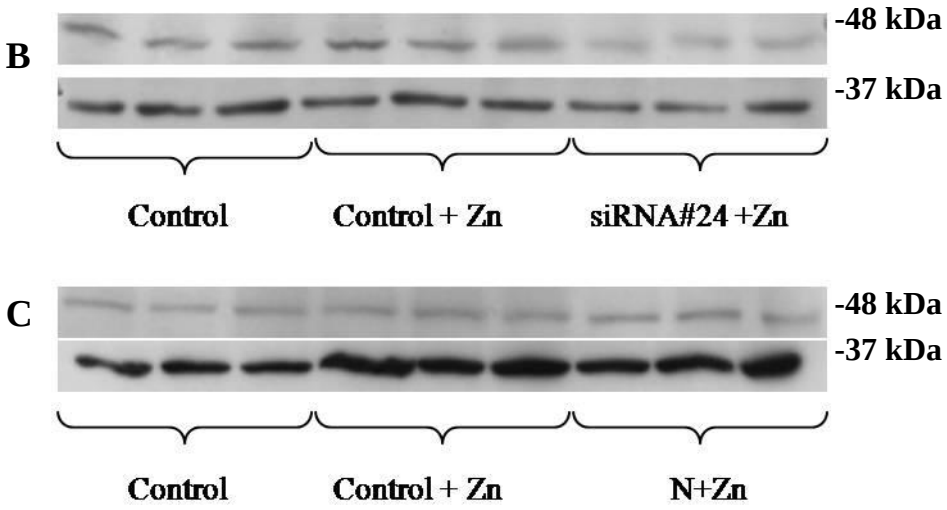

Figure 3.22.B. Representative Western blots of ZnT-4 (upper bands) and loading control (GAPDH; lower bands) for H4-APP cells treated with a gene specific siRNA #24 followed by Zn exposure. **C**. Representative Western blots of ZnT-4 (upper bands) and loading control (GAPDH; lower bands) for H4-APP cells treated with a non gene-specific siRNA sequence, negative control (N), followed by Zn exposure.

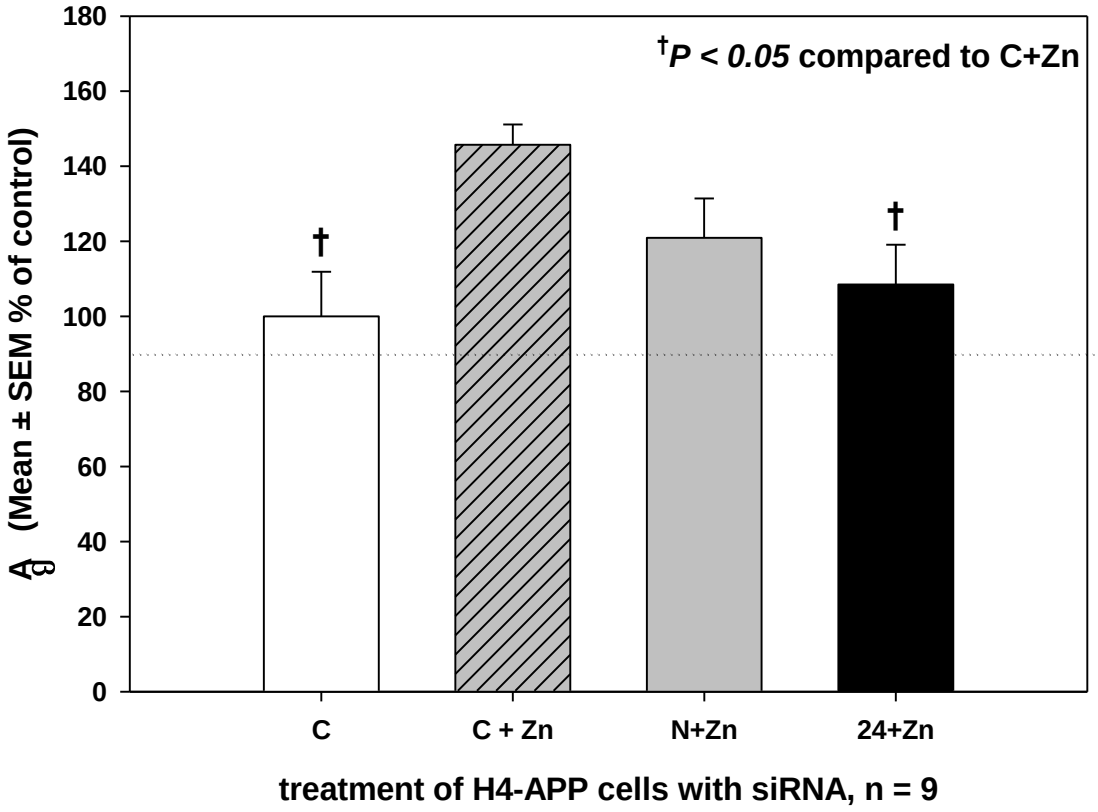

Figure 3.23. Reduction of ZnT-4 protein levels led to significantly ($P < 0.05$) decreased Aβ concentrations in the cell medium. Aβ levels expressed as mean ± S.E.M. (% of controls, C), n = 9. Medium from control cells treated with $ZnSO_4$ (C+Zn) had significantly ($P < 0.05$) increased Aβ levels compared to control cells without $ZnSO_4$ treatment (C). Medium from cells treated with siRNA #24 had significantly ($P < 0.05$) decreased Aβ concentrations compared to those of C+Zn cells. Treatment of cells with a non gene-specific siRNA sequence followed by $ZnSO_4$ treatment (N+Zn) did not result in significant ($P < 0.05$) changes in Aβ levels compared to C+Zn.

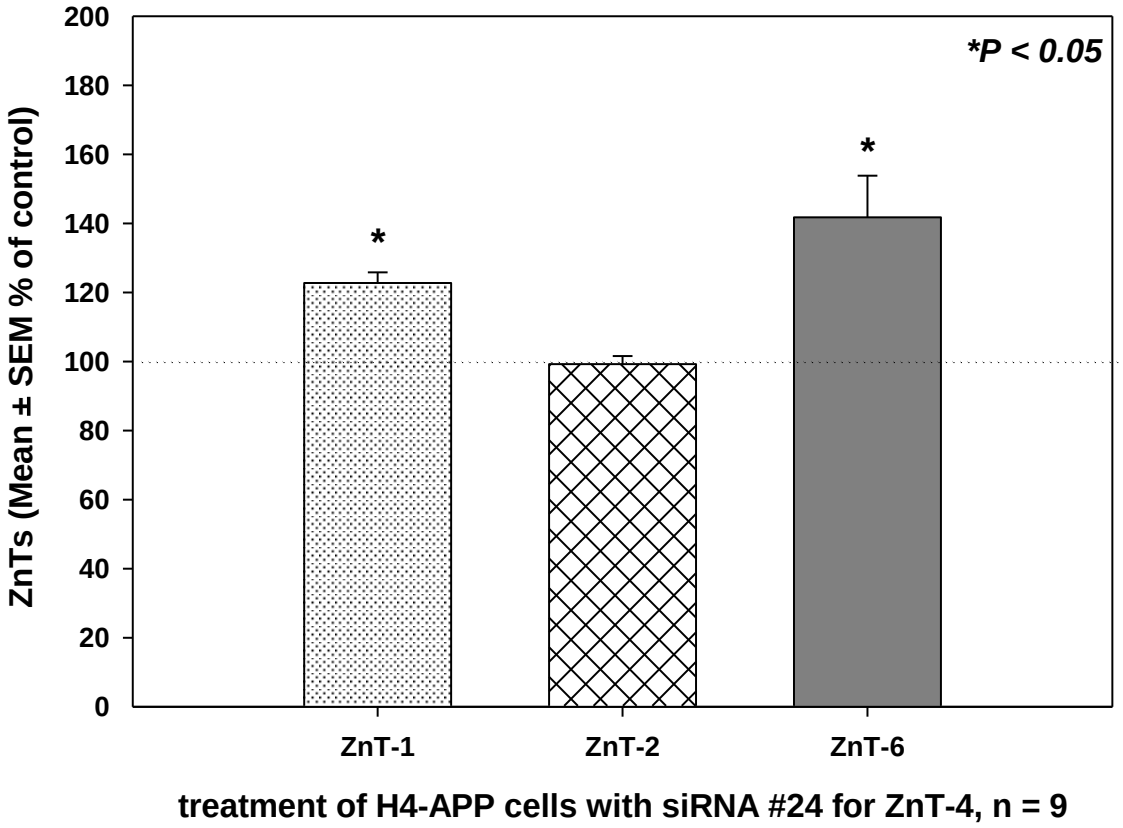

Figure 3.24. siRNA mediated reduction of ZnT-4 caused significant ($P < 0.05$) increases in ZnT-1 and ZnT-6 protein levels. ZnT levels expressed as mean ± S.E.M. (% of controls that were not treated with siRNA, shown as (C+Zn), n = 9.

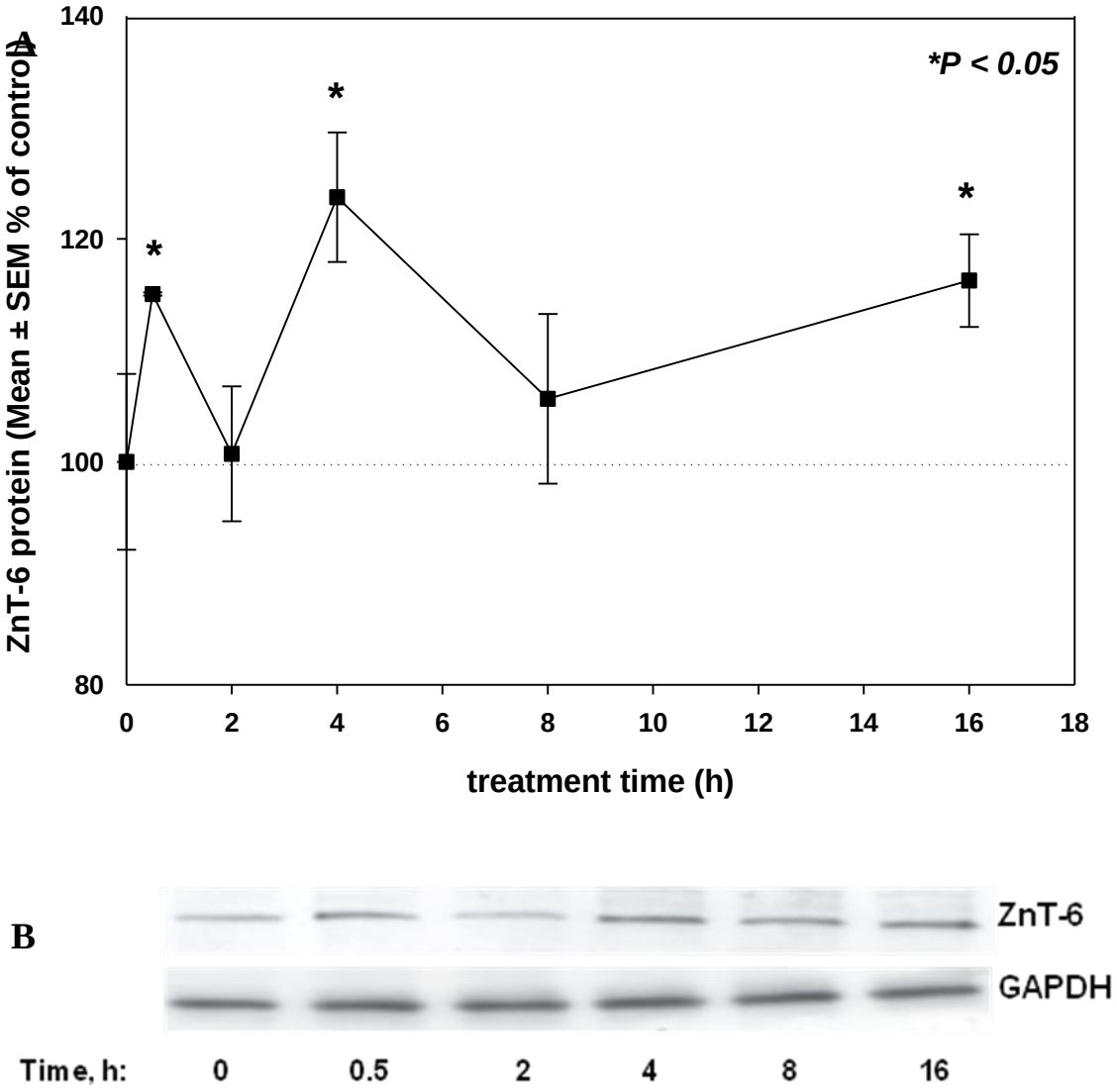

Figure 3.25. Temporal profile of ZnT-6 protein in H4-APP cells treated with 50 μM $ZnSO_4$. **A.** ZnT-6 levels are expressed as mean ± S.E.M. (% of untreated control), n = 6. **B**. Representative Western blots of ZnT-6 (upper bands) and loading control (GAPDH; lower bands) in H4-APP cells.

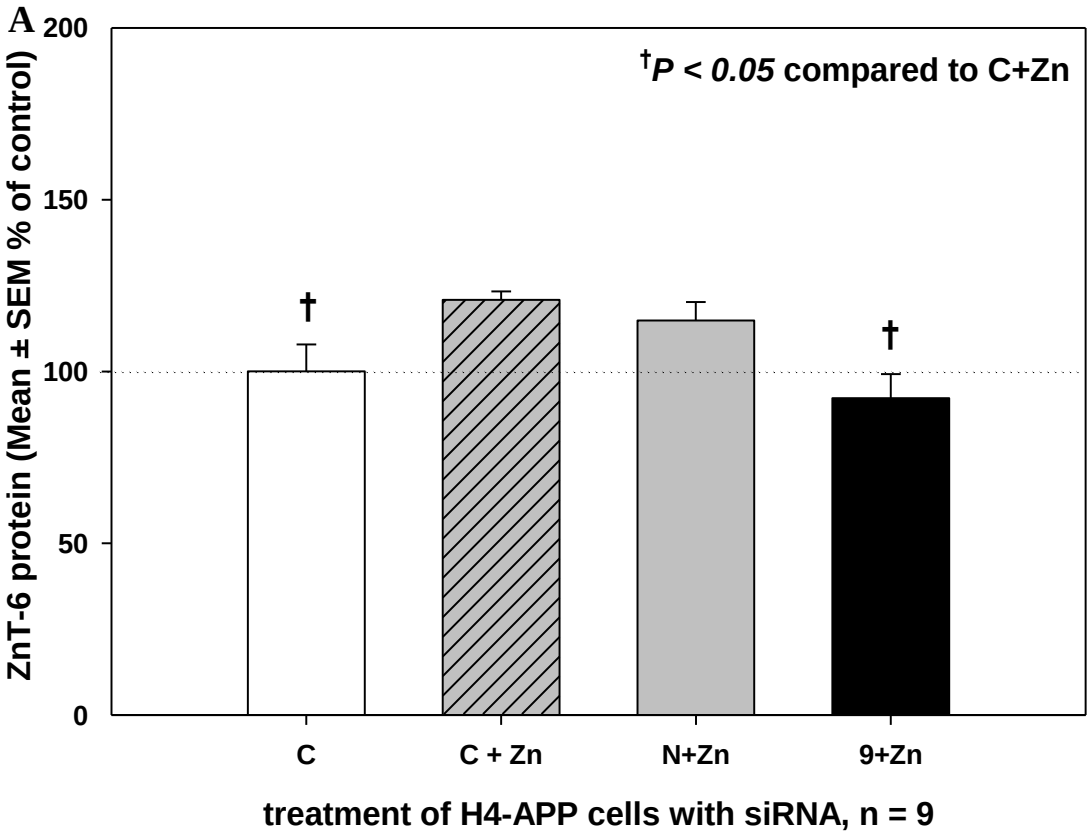

Figure 3.26. Use of a ZnT-6 siRNA was effective in reducing ZnT-6 protein levels. **A.** ZnT-6 protein levels expressed as mean ± S.E.M. (% of controls, C), n = 9. ZnT-6 sequence specific siRNA #9 led to a significant ($P < 0.05$) decrease in ZnT-6 protein levels following 24 h transfection compared to controls treated with $ZnSO_4$ but not siRNA, C+Zn. Treatment of cells with ZnT-6 non gene-specific siRNA sequence followed by $ZnSO_4$ treatment (N+Zn) did not result in significant changes in ZnT-6 levels compared to C+Zn.

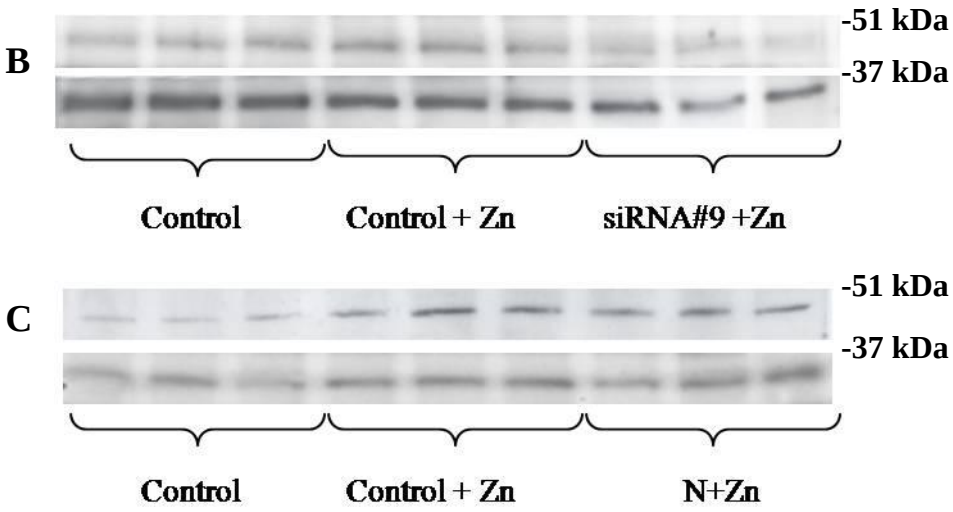

Figure 3.26.B. Representative Western blots of ZnT-6 (upper bands) and loading control (GAPDH; lower bands) for H4-APP cells treated with a gene specific siRNA #9 followed by Zn exposure. **C**. Representative Western blots of ZnT-6 (upper bands) and loading control (GAPDH; lower bands) for H4-APP cells treated with a non gene-specific siRNA sequence, negative control (N), followed by Zn exposure.

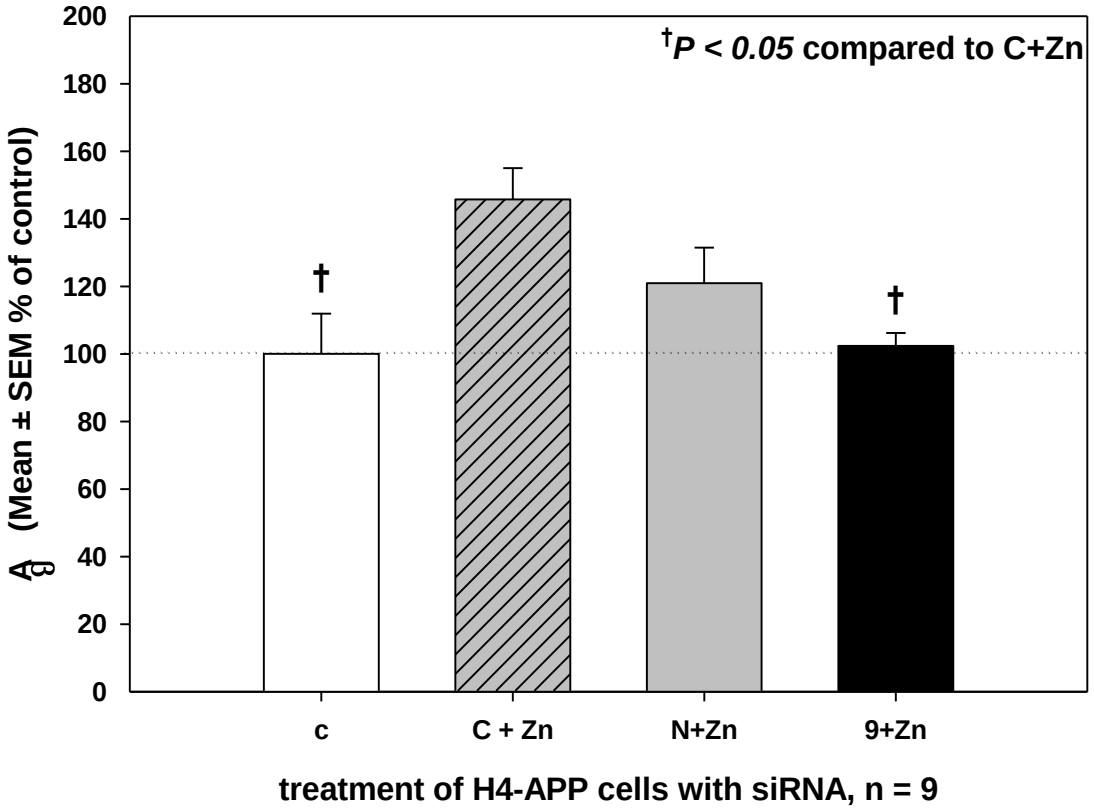

Figure 3.27. Reduction of ZnT-6 protein levels led to significantly ($P < 0.05$) decreased Aβ concentrations in the cell medium. Aβ levels expressed as mean ± S.E.M. (% of controls, C), n = 9. Medium from control cells treated with $ZnSO_4$ (C+Zn) had significantly ($P < 0.05$) increased Aβ levels compared to control cells without $ZnSO_4$ treatment (C). Medium from cells treated with siRNA #9 was characterized by significantly ($P < 0.05$) decreased Aβ levels compared to those of C+Zn cells. Treatment of cells with a non gene-specific siRNA sequence followed by $ZnSO_4$ treatment (N+Zn) did not result in significant ($P < 0.05$) changes in Aβ levels compared to C+Zn.

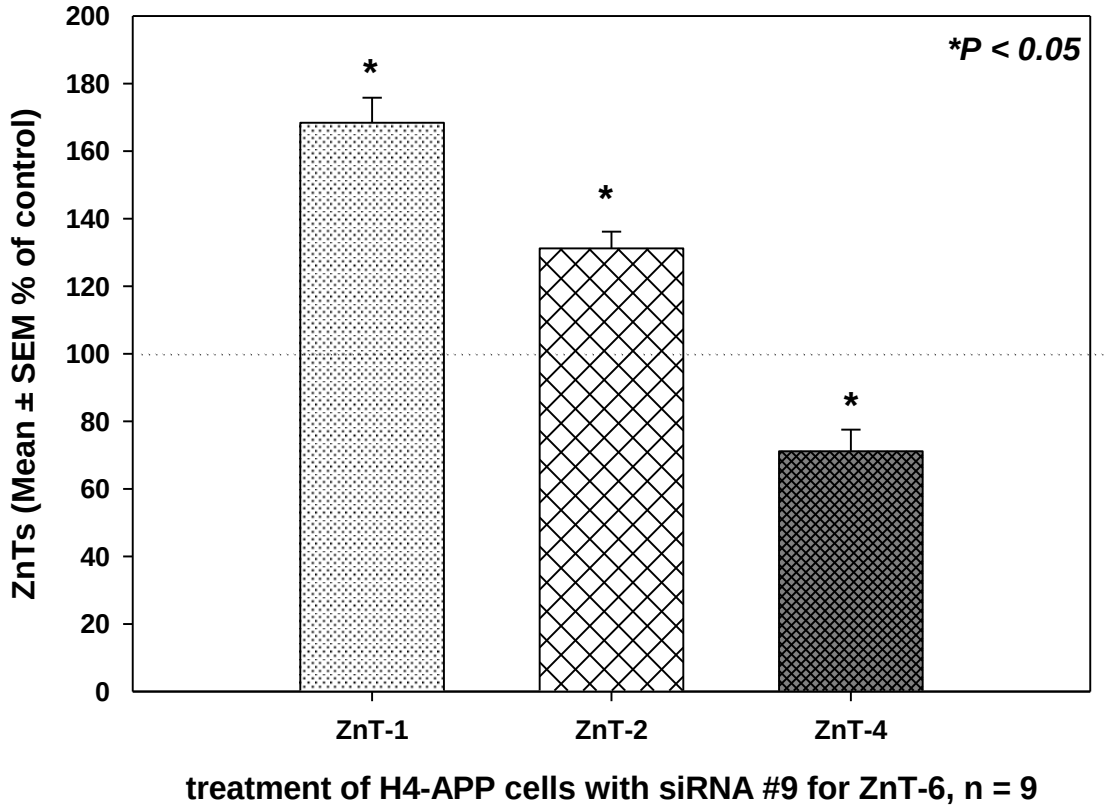

Figure 3.28. siRNA mediated reduction of ZnT-6 caused significant increases ($P < 0.05$) in ZnT-1 and ZnT-2, but a significant decrease ($P < 0.05$) in ZnT-4 protein levels. ZnT levels expressed as mean ± S.E.M. (% of controls that were not treated with siRNA, shown as C+Zn), n = 9.

CHAPTER FOUR

Discussion

4.1 Alterations in levels of ZnT-1, ZnT-4 and ZnT-6 in the brain of subjects with preclinical Alzheimer's disease (PCAD)

Alzheimer's disease (AD) affected 4.5 million Americans in 2000 (Hebert et al., 2003), and it is the eighth leading cause of death in the United States (Hoyert et al., 2006). Estimates suggest ~3% of Americans between ages 65 and 74, 19% between ages 75 and 84, and 47% over the age of 85 suffer from AD and the disease will affect 13 million by 2050, unless preventive strategies are developed (Hebert et al., 2003). Studies of subjects through the progression of the disease, especially subjects with early stages of AD, have been of considerable interest because they must benefit from development of preventive measures and/or therapeutic strategies in AD.

Disruptions of Zn homeostasis have been implicated in the pathogenesis of AD since the early 1980's (reviewed Bush et al., 1995; Cuajungco and Faget, 2003; Maynard et al., 2005; Stefanidou et al., 2006; Lovell, 2009). There are three pools of Zn in the brain: a protein bound pool, an ionic pool of free or loosely bound ions, and a vesicular pool that is released during neurotransmission (Frederickson, 1989). The chelatable, vesicular pool of Zn is present in the hippocampus, amygdala, visual, and somatosensory cortices (Frazzini et al., 2006). In gray matter Zn concentrations vary from 50 to 200 μM (Ehmann et al., 1986). During neurotransmission concentrations of Zn can reach from ~ 0.5 μM at the basal level (Assaf and Chung, 1984) to ~ 300 μM in the synaptic cleft (Maynard et al., 2005). Although several studies suggest Zn levels are increased in AD brain compared to normal healthy brain (Andrasi et al., 1995; Deibel et al., 1996; Danscher et al., 1997; Cornett et al., 1998; Lovell et al., 1998; Miller et al., 2006; Religa et al., 2006), the reason for these elevations and their direct role in the pathogenesis of AD remain unclear. One potential reason for alterations of Zn in AD is the disruption of proteins responsible for Zn homeostasis.

In general, Zn concentrations at the cellular level are tightly regulated by three families of Zn binding proteins: members of iron-regulated transporter proteins (ZIP) family, metallothioneins (MTs), and Zn transporter proteins (ZnTs) (Palmiter and Huang,

2004; Cousins et al., 2006). In this dissertation levels of ZnTs were analyzed as a starting point in the study of Zn homeostatic proteins, and we plan to address roles of ZIPs and MTs in AD in the future research. Our previous studies demonstrated alterations of ZnT-1, ZnT-4, and ZnT-6 in vulnerable brain regions of subjects with mild cognitive impairment (MCI), early and late stage Alzheimer's disease (AD) and suggest that disruptions of Zn homeostasis may play a role in the pathogenesis of AD (Lovell et al., 2006; Lovell et al., 2006; Smith et al., 2006). In the present study we analyzed levels of ZnT-1, ZnT-4 and ZnT-6 in the brain of subjects with PCAD, a condition characterized by sufficient AD pathologic alterations in the brain detected postmortem with antemortem psychometric test scores in the normal range when corrected for age and education.

Our data show a significant decrease of ZnT-1 in the HPG of PCAD subjects compared to controls (Lyubartseva et al., 2009). We observed the same trend for MCI subjects in our previous study of ZnT-1 (Lovell et al., 2006). Because the hippocampus is one of the most vulnerable regions in AD (Yaari and Corey-Bloom, 2007), diminished ZnT-1 may be one of the earliest manifestations of the disease because ZnT-1 regulates Zn efflux from the cytosol to the extracellular space (Palmiter and Huang, 2004). A decrease in ZnT-1 levels may lead to increased intracellular Zn in HPG of PCAD and MCI brain. In a previous study, concentrations of Zn at 300-600 μM cause extensive neuronal death in cortical cell culture after 15 minutes of exposure (Frederickson et al., 2005). It is possible that neurons cannot tolerate increased intracellular Zn during early stages of AD (PCAD and MCI), and therefore increase ZnT-1 production as the disease progresses (EAD, LAD) as a compensatory response. It is also possible that elevated ZnT-1 in LAD may lead to increased extracellular Zn, which can interact with Aβ and contribute to Aβ aggregation. Positive correlations between levels of ZnT-1 and senile plaques and NFT in amygdala of AD brain supports this hypothesis (Lovell et al., 2006). Although multiple proteins are responsible for maintaining Zn balance, one could speculate that decreased ZnT-1early in disease progression may contribute to increased levels of intracellular Zn in HPG of PCAD subjects that may further result in increased levels of ZnT-4 and ZnT-6, in an attempt to sequester Zn into cellular compartments. Several *in vivo* studies showed a Zn deficient diet may lead to increased levels of Zn in

rodent brain (Takeda, 2000; Chowanadisai et al., 2005) leading to decreased ZnT-1 levels in order to maintain Zn brain levels. Demographic studies suggest elderly adults, due to malnutrition, may have reduced Zn uptake (Briefel and Johnson, 2004; Marcellini et al., 2006) and a recent study by Dong et al. showed a significant decrease of serum Zn in men with MCI compared to normal controls (Dong et al., 2008).

The subcellular location of ZnT-4 suggests a possible role for this protein in the production of toxic Aβ in AD brain. Increased ZnT-4 levels could lead to increased concentrations of Zn in endosomal or lysosomal compartments that may contribute to increased Aβ production. The study by Pasternak et al. showed formation of Aβ from the amyloid precursor protein (APP) occurs in acidic organelles, specifically endosomes or/and lysosomes (Pasternak et al., 2004). Increased ZnT-4 levels would lead to increased influx of Zn into organelles contributing to more acidic pH, which would favor cleavage of APP by β- and γ-secretases resulting in increased production of Aβ (Wilquet and De Strooper, 2004). In the current study, we observed significant changes in ZnT-4 levels only in CER (Lyubartseva et al., 2009). Although the studies by Brock et al. showed the distribution of APP and Aβ are restricted to specific cell layers in CER and hippocampus (Brock et al., 2008), there are very few plaques in CER in AD brain (Braak and Braak, 1991). Previously, we reported increased levels of ZnT-4 in HPG of EAD and LAD brain; however, we did not observe significant alterations of ZnT-4 in MCI compared to controls (Smith et al., 2006). Because CER shows minimal pathology in AD (Braak and Braak, 1991), our observations of elevated ZnT-4 in PCAD CER are somewhat surprising. The relatively small number of subjects analyzed may explain our observations. Further study is needed to evaluate the possible role of ZnT-4 in PCAD.

The first study of ZnT-6 expression in AD brain demonstrated an increase of ZnT-6 in HPG of MCI, EAD, and LAD subjects compared with age matched NC subjects (Smith et al., 2006). Similarly, our current data indicate a significant elevation of ZnT-6 in HPG and CER of PCAD subjects compared to NC subjects (Lyubartseva et al., 2009). Because of the likely interplay between ZnT proteins it is possible that diminished ZnT-1 leads to increased ZnT-6 levels in PCAD. ZnT-6 is localized in the trans-Golgi network (TGN), a likely site for APP cleavage by the γ-secretase complex, and the presence of elevated Zn in the TGN may promote Aβ formation (Baulac et al., 2003). At the same

time, Zn binds specifically to human APP, and Zn binding to amyloid precursor protein (APP) inhibits proteolysis by α-secretase and causes Aβ aggregation (Bush et al., 1994b). This suggests increased levels of ZnT-6 and sequestration of Zn in the TGN may promote Aβ processing as well as affect normal sorting and trafficking of vital proteins and lipids in TGN. In our previous study (Lovell et al., 2006), we found increased ZnT-6 in degenerating neurons and a trend toward significant correlation between levels of ZnT-6 and Braak scores, which is based on NFT pathology in AD brain.

In summary, our data show significant changes in levels of two Zn transporters ZnT-1 and ZnT-6 in vulnerable regions of PCAD brain(Lyubartseva et al., 2009). Our results show a significant decrease of ZnT-1 and a significant elevation of ZnT-6 in HPG and correlate with our findings in MCI, suggesting similarities in Zn regulation patterns in PCAD and MCI. We speculate that PCAD and MCI may represent the same stage of AD that is characterized by similar levels of histopathology but different clinical manifestations. PCAD subjects are clinically normal, whereas MCI patients have memory decline. Morris and Price (Morris and Price, 2001) suggested that PCAD subjects may have protective factors that prevent or slow the development of dementia. Understanding of these factors would provide insight into pathology of AD and maximize chances of AD prevention. Together with observations of elevated Zn levels in AD, our data indicate that Zn regulatory proteins may be key mediating factors in PCAD pathophysiology. Future studies on Zn transporters in PCAD should attempt to clarify the role of Zn homeostasis in the prodromal stage of the disease.

4.2 ZnT-2 levels in the brain of subjects with preclinical Alzheimer's disease (PCAD), mild cognitive impairment (MCI), early (EAD) and late-stage Alzheimer's disease (LAD)

Our studies are the first to demonstrate the presence of ZnT-2 mRNA using reverse transcriptase polymerase chain reaction (RT-PCR), and protein using Western blot analysis. ZnT-2 was first described in studies of Palmiter et al., who concluded that ZnT-2 protects a cell from Zn toxicity by facilitating Zn transport into endosomal and lysosomal compartments of baby hamster kidney (BHK) cells (Palmiter et al., 1996a). Presence of the ZnT-2 transcript in human brain suggests similar protective mechanism. However, BHK cells analyzed by Palmiter's group are different from neurons and therefore our study of ZnT-2 could clarify the role of this Zn transporter in human brain.

Levels of ZnT-2 in LAD brain were significantly altered in all three brain regions studied (hippocampus and parahippocampal gyrus (HPG), superior and middle temporal gyrus (SMTG) and cerebellum (CER)) compared to NC subjects. We speculate that with the progression of the disease, Zn alterations in AD brain become more prominent, leading to pronounced changes of ZnT-2 protein levels in LAD brain, potentially contributing to the presence of SP and NFT pathology.

Among brain regions analyzed in the current study, HPG shows the most pronounced AD pathology. It is responsible for the formation of new memories (Morris, 2006) and the most affected region in AD brain (Burns and Iliffe, 2009). ZnT-2 levels in HPG of LAD brain are significantly elevated compared to NC subjects. In our previous studies we found that levels ZnT-1, ZnT-4 and ZnT-6 levels in HPG of LAD brain were also increased (Lovell et al., 2005; Lovell et al., 2006; Smith et al., 2005). Most studies showed that Zn levels were higher in HPG of AD brain compared to controls (Deibel et al., 1996; Cornett et al., 1998; Lovell et al., 1998). It could be hypothesized that as the disorder progresses, Zn concentrations in the brain increase and cells attempt to sequester or remove excessive Zn by increasing levels of ZnTs. We speculate changes in ZnT-1, ZnT-2, ZnT-4 and ZnT-6 may contribute to increased production and aggregation of Aβ, the main component of senile plaques in AD brain.

Similar to LAD, ZnT-2 in the brain of EAD subjects was significantly increased in HPG compared to age matched NC subjects. Strikingly, levels of other ZnTs: ZnT-1, ZnT-4 and ZnT-6 area were also elevated in HPG of both EAD and LAD groups (Lovell et al., 2005; Lovell et al., 2006; Smith et al., 2005). Analogous behavior of four ZnTs for EAD and LAD brains suggests similarity of Zn regulation patterns in these two stages of AD.

In contrast, we observed a trend toward a significant decrease of ZnT-2 in MCI HPG. In the current study we analyzed a relatively small number of MCI subjects and differences in ZnT-2 levels between MCI and NC HPG may become more apparent when comparing a larger cohort of subjects. ZnT-2 levels in HPG of PCAD subjects were significantly decreased compared to NC subjects. PCAD brain is characterized by less severe plaque pathology compared to EAD and LAD (Knopman et al., 2003; Galvin et al., 2005).

The subcellular location of ZnT-2 suggests a potentially important role in the formation of toxic Aβ in AD brain. Increased ZnT-2 levels could cause increased Zn sequestration in endosomal or lysosomal compartments. Elevated Zn concentrations in endosomes or lysosomes may contribute to formation of toxic Aβ. An increasing body of evidence suggests formation of Aβ from the amyloid precursor protein (APP) occurs in intracellular acidic compartments of the cell, which may be endosomes or/and lysosomes (Pasternak et al., 2004). Increased ZnT-2 levels would lead to increased influx of Zn in organelles making their pH more acidic. Acidic pH favors cleavage of APP by β- and γ-secretases which results in the production of Aβ (Wilquet and De Strooper, 2004). We speculate that increased ZnT-2 in HPG and SMTG of LAD may lead to Zn accumulation in endosomal/lysosomal compartments and contribute to formation of Aβ.

In our previous study we observed a significant decrease of ZnT-1 in HPG of PCAD and MCI compared to NC subjects (Lyubartseva et al., 2009) . It is possible that simultaneous decreases in ZnT-1 and ZnT-2 levels in early stages of the disease are part of the same mechanism by which cells are trying to retain Zn in cytosol. We suggest that ZnT alterations of in brains of PCAD and MCI subjects may result from Zn deficiency. Zn deficiency as a cause of AD was one of the first etiological AD hypothesis proposed (Burnet, 1981). In addition there are contemporary studies, which suggest Zn deficiency

may be associated with AD (Briefel and Johnson, 2004; Lovell et al., 2006; Marcellini et al., 2006; Dong et al., 2008).

The SMTG is the area of human brain that is responsible for language (Price, 2000). Language impairment is one of the clinical symptoms of AD, especially during late stages (Anonymous, 1997; Price, 2003). Levels of ZnT-2 are significantly elevated in SMTG of subjects with PCAD, MCI and LAD compared to NC subjects. In contrast, there were no significant alterations of ZnT-2 levels in MCI SMTG compared to controls. These observations provide an additional support to the idea of altered Zn homeostasis in AD brain compared to healthy brain.

CER shows relatively little neuropathology in AD (Braak and Braak, 1991). However, studies indicated that clinical symptoms of AD (Newberg et al., 2003) as well as pathological features including deposits of amyloid (Mann et al., 1996) and activated microglia (Mattiace et al., 1990) are associated with CER.

The present data show significantly depleted ZnT-2 levels in CER of LAD subjects compared to age matched NC subjects, similar to the trend we observed for ZnT-4 (Smith et al., 2006). Both ZnT-2 and ZnT-4 transport Zn into endosomal or lysosomal compartments, which may be a key point in understanding our observations. Clearly, studies of subcellular locations of both ZnT-2 and ZnT-4 are required to understand roles of these proteins in AD brain.

In conclusion, we detected ZnT-2 in human brain and analyzed levels of ZnT-2 protein in four stages of AD: PCAD, MCI, EAD and LAD compared to NC subjects. Our data show that as the disease progresses, there are more pronounced changes of ZnT-2 levels in AD brain. Curiously, PCAD subjects show alterations in ZnT-2 levels in two areas (HPG and SMTG) compared to only one area (SMTG) for MCI subjects. Thus, ZnT-2 alterations correlate with the severity of pathology but not clinical manifestations of AD for these two stages. HPG, the most vulnerable region to AD pathology, shows alterations of ZnT-2 levels for all stages of AD. We speculate that abnormal fluctuations of ZnT-2 affect the distribution of Zn in neurons and therefore may affect Aβ production in AD brain. It is important to confirm and extend the above findings about the role of ZnT-2 in Aβ production.

4.3 A role for ZnT-1, ZnT-2, ZnT-4 and ZnT-6 in amyloid beta (Aβ) peptide processing

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 preclinical AD (PCAD), mild cognitive impairment (MCI), early AD (EAD) and late stage AD (LAD) compared to age-matched normal control (NC) subjects (Lovell et al., 2006; Lovell et al., 2006; Smith et al., 2006; Lyubartseva et al., 2009). To test the hypothesis that changes in Zn sequestration in specific subcellular organelles by ZnT proteins affects production of amyloid beta peptide (Aβ) we investigated the relationship between levels of ZnT-1, ZnT-2, ZnT-4, ZnT-6 and concentrations of Aβ secreted into media of H4 human neuroglioma cells transfected to overexpress amyloid precursor protein (H4-APP). Our results show treatment of H4-APP cells with 50 μM Zn led to significant increases in Aβ secretion into the medium and that siRNA mediated decreases of ZnT-2, ZnT-4, and ZnT-6 in cultures treated with Zn lead to significantly decreased total Aβ in the medium.

We speculate that functions and subcellular location of analyzed ZnTs play an important role in the formation of Aβ from APP via cleavage by β- and γ-secretases. From previous studies, we know the cellular locations of ZnTs (Palmiter et al., 1996a; Huang and Gitschier, 1997; Huang et al., 2002; Ohana et al., 2006). However, exact subcellular locations of β- and γ-secretases as well as the site of Aβ production are not well established. It is generally believed that APP cleavage by secretases and intracellular formation of Aβ take place in acidic vesicles: endosomes, lysosomes, Golgi/TGN (Sambamurti et al., 1992; Huse et al., 2000; Petanceska et al., 2000; Sudoh et al., 2000; Huse et al., 2002; Baulac et al., 2003; Kinoshita et al., 2003b; Cataldo et al., 2004; Pasternak et al., 2004; Vetrivel et al., 2004; Wilquet and De Strooper, 2004; Koh et al., 2005; Hu et al., 2007; Liu et al., 2007).

To determine which ZnTs among those studied were the most sensitive indicator of increased extracellular Zn concentrations, we analyzed ZnT-1, ZnT-2, ZnT-4, and ZnT-6 levels following exposure to 50 μM Zn. H4-APP cells treated with Zn for 16 hours showed increases in ZnT levels for all proteins studied, except ZnT-2. Bobilya et al. analyzed longitudal changes in ZnT-2 protein levels and reported that ZnT-2 was

significantly increased at 12 and 24 h during 50 μM Zn treatment in porcine brain capillary endothelial cells (BCEC). Our results show that in H4-APP cells ZnT-2 is not induced by 50 μM Zn treatment during the course of 16 hours. We speculate that ZnT-2 response to Zn may be cell specific and vary between BCEC and H4-APP cells. Although our current ZnT-2 data appear at odds with the results of the previous study, they are consistent with ZnT-1 behavior described by Bobilya et al. (Bobilya et al., 2008). Based on results from other groups (Palmiter and Huang, 2004), we anticipated that ZnT-1 would be the most sensitive to elevated Zn, and our data supported the hypothesis. Curiously, ZnT-4 and ZnT-6 responses appear to be cyclic and to be in opposite phases. Our data show when ZnT-4 levels are at maximum, ZnT-6 levels are at the baseline and vice versa. It is possible that these two ZnTs work in concert to evenly distribute Zn during periods of increased concentrations.

In the majority of previous studies of ZnT functional mechanisms researchers analyzed overexpression of ZnT-1 protein (Palmiter and Findley, 1995; Segal et al., 2004). However, as Ohana et al. suggested, an overexpression may interfere with the natural function of the protein (Ohana et al., 2006). Therefore, we chose to use silencing of ZnT proteins in the current study. We used short interfering RNAs (siRNA) to silence ZnTs' gene expression followed by analysis of levels of Aβ in the cell medium. siRNA silencing is a method in which siRNA triggers the degradation of targeted mRNAs of a specific gene, therefore suppressing its expression and consequently lowering its protein levels (Leung and Whittaker, 2005). siRNA is a part of a naturally occurring catalytic gene regulation system which acts as a defense mechanism against pathogens (Wang et al., 2004b). The siRNA approach is appropriate for suppression of ZnT protein levels because siRNA offer high specificity, scalability and an excellent experimental reproducibility in suppressing endogenous gene expression in mammalian cell systems (Wang et al., 2004b). In addition, the previous study by Ohana et al. demonstrated an effectiveness of siRNA method in ZnT-1 silencing in rat cortical neurons (Ohana et al., 2006).

ZnT-1 is abundant in areas rich in synaptic Zn and has been proposed to have a protective role against Zn cytotoxicity in the nervous system (Sekler et al., 2002). Our previous studies of ZnT-1 in AD brain showed a significant decrease ($P < 0.05$) of ZnT-1

in hippocampus and parahippocampal gyrus (HPG) of PCAD and MCI subjects (Smith et al., 2006; Lyubartseva et al., 2009). Our present data show that decreasing ZnT-1 protein did not significantly affect levels of Aβ or other ZnTs (ZnT-2, ZnT-4 and ZnT-6). Therefore, it is possible that changes ZnT-1 alone may not affect Aβ production. We speculate that, since ZnT-1 is responsible for the transport of Zn to the extracellular space, it may not be involved in the intracellular process of Aβ formation. This conclusion led us to investigate roles of other ZnTs which regulate levels of intracellular Zn by transferring Zn from cytosol to organelles. Also, it is worth noting that the treatment of H4-APP cells, not transfected with siRNAs, with 50 μM Zn in the course of 16 h caused a significant ($P < 0.05$) increase in Aβ levels. In our opinion, this observation indicates the connection between Zn and Aβ.

ZnT-2 was first described by the studies of Palmiter et al. (Palmiter et al., 1996a; Palmiter et al., 1996b), who concluded ZnT-2 protects from Zn toxicity by facilitating Zn transport into endosomal and lysosomal compartments of the cell (Palmiter et al., 1996a). The presence of the ZnT-2 transcript in human brain suggests similar protective mechanisms. In the previously described project we detected ZnT-2 mRNA in human brain tissue using RT-PCR, and showed a significant decrease ($P < 0.05$) in ZnT-2 levels in HPG of PCAD subjects compared to ZnT-2 levels in HPG of age matched NC subjects. In addition, ZnT-2 was significantly ($P < 0.05$) elevated in HPG of EAD and LAD brains compared to NC. In the present study we found that the reduction of ZnT-2 protein levels leads to decreased Aβ concentrations in the cell medium. The location of ZnT-2 (Palmiter et al., 1996a) indicates its potential role in the formation of toxic Aβ in AD brain. Kinoshita et al. demonstrated that β-secretase cleaves APP in endosomes (Kinoshita et al., 2003b). In addition, Pasternak et al. showed that in neurons and astrocytes γ-secretase cleaves APP in lysosomes (Pasternak et al., 2004). One of the requirements of the APP cleavage by β- and γ-secretases is the low pH (Wilquet and De Strooper, 2004). The presence of Zn ions would likely shift the equilibrium to a more acidic pH. Therefore, siRNA mediated decreased concentration of Zn ions provides less favorable conditions for β- and γ-secretases processing and could possibly lead to decreased production of toxic Aβ.

Similar to ZnT-2, ZnT-4 is located on lysosomal and endosomal compartments and functions to sequester Zn in these compartments (Huang and Gitschier, 1997). Our previous studies show elevated levels of ZnT-4 in HPG of EAD and LAD subjects (Smith et al., 2006). In the present study, we detected a simultaneous decrease of ZnT-4 protein levels in ZnT-2 siRNA transfected cells. We were somewhat surprised by the latter finding, because we expected an opposite effect: once we silence ZnT-2, other ZnT levels might increase to redistribute Zn between the cytosol and intracellular compartments. Previous studies referred to the similarity between ZnT-2 and ZnT-4 protein sequences and we first assumed that ZnT-2 specific siRNAs may also affect ZnT-4 protein levels. However, ZnT-2 siRNA sequence does not overlap with the ZnT-4 sequence. Also, we did not observe any significant ($P < 0.05$) change in ZnT-2 levels when we conducted ZnT-4 silencing. Thus, we consider that there may be other factors (i.e. altered levels of ZIPs or MTs) which affect protein levels of ZnTs during siRNA experiment and, therefore, the mechanism of ZnTs' regulation may be more complicated compared to the one that we proposed initially.

As we expected, siRNA silencing of ZnT-4 led to decreased levels of Aβ in the cell medium. We speculate that, because of analogous subcellular locations, ZnT-2 and ZnT-4 may affect the mechanism of Aβ production similarly. We also found corresponding increases in ZnT-1 and ZnT-6 protein levels during ZnT-4 siRNA experiments. It is possible that a decrease in ZnT-4 leads to increased concentrations of Zn in cytosol, and, in order to prevent Zn from further rising to toxic levels, the cells attempt to sequester Zn to other organelles, TGN for ZnT-6, or extracellular space for ZnT-1, via the regulation of ZnTs other than ZnT-4. Previously, we reported increased levels of ZnT-4 in HPG of EAD and LAD brain, both characterized by the abundance of Aβ plaques (Smith et al., 2006). Therefore, we speculate that an opposite phenomenon may occur and siRNA reduction of ZnT-4 as well as ZnT-2 resulted in diminished Aβ levels.

Previous studies demonstrated an increase in ZnT-6 in HPG of PCAD, MCI, EAD, and LAD subjects compared with age matched NC subjects (Lovell et al., 2006; Smith et al., 2006; Lyubartseva et al., 2009). ZnT-6 is localized in the TGN, a likely site for APP cleavage by the γ-secretase complex, and may promote Aβ formation (Baulac et

al., 2003). At the same time, Zn binds specifically to human APP, and Zn binding to amyloid precursor protein (APP) inhibits proteolysis by α-secretase and causes Aβ aggregation (Bush et al., 1994b). This suggests increased levels of ZnT-6 and sequestration of Zn in the TGN may promote Aβ processing as well as affect normal sorting and trafficking of vital proteins and lipids in TGN. In our previous studies, we found increased ZnT-6 in degenerating neurons and a trend toward significant correlation between levels of ZnT-6 and Braak scores, which are based on the neurofibrillary tangle (NFT) pathology in AD brain (Lovell et al., 2006; Lyubartseva et al., 2009).

In the present study we also analyzed the effects of ZnT-6 reduction on Aβ production. Based on previous studies (Lovell et al., 2006; Smith et al., 2006; Lyubartseva et al., 2009) we hypothesized that reductions of ZnT-6 may lead to a corresponding decrease in Aβ secretion. Our data support this hypothesis and show that decreasing ZnT-6 not only resulted in significantly ($P < 0.05$) decreased levels of Aβ, but also affected levels of all other ZnTs (ZnT-1, ZnT-2 and ZnT-4). We observed that levels of ZnT-1 and ZnT-2 were increased, and the levels ZnT-4 were decreased. We explain corresponding increases of two ZnTs by the existence of the compensatory mechanism described earlier in the discussion of ZnT-4 silencing. We speculate that a decrease in ZnT-4 levels during ZnT-6 silencing may result from ZnT-4 and ZnT-6 occurring in opposite phases (temporal profiles of ZnT-4 and ZnT-6). ZnT-6 siRNA sequences did not overlap with ZnT-4 protein sequence. However, we did not see an opposite effect i.e. significant ($P < 0.05$) decrease in ZnT-6 levels during ZnT-4 silencing experiments. Instead, we found an elevation of ZnT-6 protein levels after ZnT-4 siRNA transfection. Further studies are needed to understand the nature of interactions, if such exist, between ZnT-4 and ZnT-6.

In summary, our results suggest siRNA mediated reductions of ZnT-2, ZnT-4, and ZnT-6 lead to decreased levels of Aβ, neurotoxic protein which forms senile plaques in AD brain. Attenuations of ZnT-2, ZnT-4, and ZnT-6 protein levels probably result in altered Zn concentrations in the cell and that subsequently leads to changes in Aβ levels. We speculate that the function and location of ZnTs in the cell indicate that these proteins may be involved in the intracellular Aβ production. Together with our previous studies of

ZnTs in AD brain, current data a possible link between ZnT mediated Zn regulation and Aβ formation in AD.

CHAPTER FIVE

Conclusion

Alzheimer's disease (AD), one of the major causes of disability and mortality in Western societies, is a growing public health concern, because of a rising global prevalence, the lack of effective diagnostic procedures and effective treatment options (Yaari and Corey-Bloom, 2007). One etiologic hypothesis suggested for the pathogenesis of AD that has received considerable interest is the metal hypothesis, particularly the role of alterations of Zn (Markesbery and Ehmann, 1994; Cuajungco and Faget, 2003). Numerous studies show elevations of Zn in brains of subjects with AD compared to age-matched healthy controls, particularly in areas vulnerable to AD pathogenesis: hippocampus and amygdala (Deibel et al., 1996; Danscher et al., 1997; Lovell et al., 1998; Religa et al., 2006).

The role of Zn in AD pathogenesis is controversial and is inferred from experimental models (Frederickson et al., 2005). The effect of Zn on protein/peptide alterations observed in AD has been extensively studied (Wilquet and De Strooper, 2004). Pathologically the AD brain is characterized by accumulation of amyloid beta (Aβ) peptide which results from a proteolytic cleavage of amyloid precursor protein (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 (Bush et al., 1995). 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 (Wilquet and De Strooper, 2004). It was also observed by Bush et al. (Bush et al., 1993; Bush et al., 1994b) 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 (Bush et at., 1995; Cuajungco and Faget, 2003; Frederickson et al., 2005). Numerous reports demonstrate that Zn can act as a potential mediator of neuronal degeneration (reviewed in Frazzini et al., 2006). Additionally, loss

of neurons in epilepsy, ischemia, and traumatic brain injury is characterized by elevations of neuronal Zn (Frederickson et al., 2005).

At the cellular level, Zn concentrations are controlled by three families of Zn binding proteins: zinc-regulated and iron-regulated transporter proteins (ZIP), metallothioneins (MT), and Zn transporter (ZnT) proteins (Palmiter and Huang, 2004; Cousins et al., 2006). To understand the mechanism of Zn disruptions in AD we studied the possible role of ZnTs which are expressed in human brain (ZnT-1, ZnT-2, ZnT-4 and ZnT-6) in the progression of AD. Based on clinical criteria and pathological features there are three stages of AD severity: mild cognitive impairment (MCI), early (EAD) and late stage AD (LAD). Recently, a preclinical stage of AD (PCAD) has been proposed. During life, AD clinical manifestations in PCAD subjects were not detected, but postmortem brains are characterized by significant AD neuropathology. It is possible that PCAD stage may be followed by the development of MCI and AD (Knopman et al., 2003; Fagan et al., 2005; Galvin et al., 2005).

The hippocampus is responsible for the formation and consolidation of memory (Morris, 2006), and is one of the most vulnerable regions in AD pathogenesis (Braak and Braak, 1991). In addition, several studies found altered distribution of Zn in the hippocampus of AD subjects compared to controls (Deibel et al., 1996; Cornett et al., 1998; Lovell et al., 1998; Religa et al., 2006). The summary of current and previous measurements of ZnT protein levels in hippocampus/parahippocampal gyrus (HPG) of AD brain compared to normal control (NC) subjects is shown in the Table 5.1. Current data suggest the HPG of AD subjects at any stage of severity shows profound alterations of ZnTs compared to age matched NC subjects. Using Western blot analysis and immunohistochemistry we detected a significant decrease of ZnT-1 in the HPG of PCAD subjects compared to NC subjects (Lyubartseva et al., 2009). In previous studies from our laboratory we observed the same trend for MCI subjects (Lovell et al., 2006), suggesting similarities of Zn regulation patterns for these two stages of AD. Intriguingly, ZnT-1 levels are increased in HPG of EAD and LAD subjects (Lovell et al., 2006). The mechanism of ZnT-1 regulation, proposed by Andrews et al., states that cytoplasmic Zn binds the Zn-finger domain of metal response element-binding transcription factor-1 (MTF-1) (Laity and Andrews, 2007). Once binding occur, MTF-1 trans-locates to the

nucleus, followed by a change in conformation, where it binds the metal response element (MRE) in the gene for ZnT-1 and induces ZnT-1 expression (Andrews, 2001). Thus, high concentrations of Zn in the cytosol, caused by decreased ZnT-1 during early stages, may lead to elevated levels of ZnT-1 during late stages of AD. We speculate increased ZnT-1 in LAD may lead to increased extracellular Zn, which can interact with Aβ and contribute to Aβ aggregation as described by Bush et al. (Bush et al., 1994b; Bush et al., 1994a). Positive correlations between levels of ZnT-1 and senile plaques and NFT in amygdala of AD brain support this hypothesis (Lovell et al., 2006).

Until recently, the reason for decreased ZnT-1 during early stages of AD was unclear. However, *in vivo* animal studies by Zhang et al. (2008b), our group (unpublished results) and Dong et al. (2008) suggest there may be a link between Zn deficiencies, Zn serum levels and Zn brain levels in AD. Zhang et al. demonstrated a statistically significant increase of ZnT-1 in the hippocampus of APP/PS1 mouse, a transgenic animal model of Aβ deprivations, induced by a Zn deficient diet (Zhang et al., 2008b). Our unpublished data suggest decreased ZnT-1 levels in mouse brain may be induced by a Zn deficient diet. Demographic studies show the elderly population in general has insufficient Zn uptake and that there may be a relationship between low extra-parenchymal Zn levels and cognitive decline (Briefel and Johnson, 2004; Marcellini et al., 2006). Zn deficiency could be easily corrected by Zn containing food supplements and presents a potential target for AD therapy. Dong et al. using inductively coupled plasma-mass spectrometry (ICP-MS) quantified serum Zn levels from living mild to moderate AD, MCI patients and NC subjects, and concluded that men with MCI had a statistically significant decrease of serum Zn compared to women with MCI and NC men subjects (Dong et al., 2008). These data may explain the loss of brain ZnT-1 observed in our studies. The possible interpretation is that during early stages of AD patients experience a systemic Zn deficiency which causes increased brain Zn via down regulation of ZnT-1. To maintain Zn stores in the brain neurons try to retain more Zn in the cytosol by decreasing levels of ZnT-1 which transport Zn from the cytosol to extracellular compartments. To confirm this hypothesis it would be beneficial to analyze Zn levels in brains of MCI subjects as well as in brains and body fluids of PCAD subjects.

In our second project we detected ZnT-2 in human brain, apparently the fifth ZnT along with ZnT-1, ZnT-3, ZnT-4 and ZnT-6 expressed in the brain. This protein was previously characterized in non-neuronal cell types and was proposed a role to protect these cells from Zn toxicity by facilitating Zn transport into an endosomal/lysosomal compartment (Palmiter et al., 1996a). The purpose of our research was to evaluate the expression of ZnT-2 throughout the progression of AD. HPG of PCAD, MCI, EAD and LAD subjects showed alterations of ZnT-2 compared to NC (Table 5.1). It is striking that ZnT-1 and ZnT-2 follow the same patterns in different stages of AD, that is: significant decreases in PCAD and MCI HPG and increases in HPG of subjects with late stages of AD, EAD and LAD (Table 5.1). We speculate that low ZnT-2 levels in PCAD compared to NC brain, considering that ZnT-2 sequesters Zn from cytosol to intracellular endosomal/lysosomal compartments, together with decreased ZnT-1 lead to increase of cytosolic Zn. Abnormally high concentrations of Zn accumulated in cytosol during early stages may be potentially toxic to neurons. Therefore all ZnTs, responsible for removal of Zn from the cytosol, become elevated as the disease progresses (Table 5.1).

To understand how ZnT alterations in AD brain compared to NC may be linked to increased Aβ production, we carried out the third project, the main purpose of which was to investigate the relationship between protein levels of ZnT-1, ZnT-2, ZnT-4, ZnT-6 suppressed by siRNAs and concentrations of Aβ in the media of H4 human neuroglioma cells (H4-APP) transfected to overexpress APP. We chose to use this cell line to maximize the amount of secreted Aβ and thus improve detection (Liu et al., 2007). Although it is possible that overexpression of APP may affect the cellular localization of APP and thus potentially its interaction with various ZnT proteins (Ohana et al., 2006), the H4-APP cell culture model is appropriate for our studies because of its stability and short usable life span (Krex et al., 2001). Based on our results, decreased ZnT-1 observed during early stages of AD (Table 5.1) alone may not be enough to promote elevations of other ZnTs and lead to increased Aβ production because siRNA suppression of ZnT-1 did not affect Aβ levels secreted to cell media. Our results suggest suppression of ZnT-4 and ZnT-6 led to decreased Aβ levels in cell media compared to controls. We speculate the opposite may be true; thus, increased levels of these ZnTs may promote increased Aβ production. Data from our current research and previous studies of ZnTs in AD brain

(Table 5.1) reflect these trends: ZnT-4 and ZnT-6 are increased throughout the progression of the disease all stages of each are accompanied increased Aβ production in AD brains compared to NC.

Overall, our results suggest ZnTs function in concert and the regulation of one ZnT affects levels of others. In future projects we will need to analyze levels of ZIP proteins and MTs in AD to understand the connection between Zn homeostatic proteins and Zn alterations in AD. ZIP proteins are of special interest because they have opposite functions compared to ZnTs - transport Zn from extracellular space or from intracellular vesicles to the cytosol. However, a lack of commercial antibodies for ZIPs presents a technical problem. There are MTs studies in aging brains but results have been conflicting (Mocchegiani et al., 2005).

Zn levels in AD brain were extensively analyzed whereas not many studies quantified extra-parenchymal Zn, especially levels of Zn in cerebrospinal fluid (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 neurofibrillary tangles (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 (Lovell et al., 1998). 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 (Zhang et al., 2008a). In contrast, the potential role of Zn in the formation of NFTs was hypothesized and supported by *in vitro* experiments (An et al., 2005) but not addressed *in vivo*.

There has been considerable interest in the regulation of ZnTs in animal models of AD (Lee et al., 2002; Zhang et al., 2008b). Recent studies by Zhang et al. (Zhang et al., 2008b) showed increased ZnT-1, ZnT-4, and ZnT-6 in the hippocampus and neocortex of APPswe/PS1dE9 mice. In addition, ZnT immunostaining was associated

with most amyloid plaques in those mice (Zhang et al., 2008b). 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. (Lee et al., 2002), 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.

The work described in this dissertation is the study of a potential role of ZnT alterations in AD. Although our data suggest a link between alterations of ZnTs and increased Aβ in the progression of AD, there is a need for further studies of Zn, 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. Based on previous studies (Lovell et al., 1998; Briefel and Johnson, 2004; Marcellini et al., 2006; Dong et al., 2008; Lovell, 2009) and current data, 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 SP. While the cause of AD still remains unknown, we hope results of this work will contribute to our understanding of the interplay among biochemical changes which involve Zn, associated with AD.

Table 5.1. Summary of ZnT protein levels in HPG of subjects with different stages of AD compared to NC subjects.

ZnT levels compared to NC / Stage of AD	ZnT-1	ZnT-2	ZnT-4	ZnT-6
PCAD	↓*	↓*	↑	↑*
MCI	↓*	↓	↑	↑
EAD	↑*	↑*	↑*	↑*
LAD	↑*	↑*	↑*	↑*

***P** < 0.05

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