Metallothionein III and S100ß modulate neuronal activity and survival

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ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia and is characterized at postmortem by the deposition of amyloid plaques and intraneuronal neurofibrillary tangles. Plaques are primarily composed of amyloid-β (Aβ) peptide, while tangles are assembled from abnormally phosphorylated microtubule associated tau protein. Both are morphologically fibrous structures and directly neurotoxic. These pathological events are accompanied by reactive gliosis and extensive neuronal loss leading to clinical dementia. The central focus of this thesis is that dysregulation of metal-binding growth factors and their relationship to astrocyte function, contributes to AD pathogenesis. These studies focus on metallothionein-III (MT-III) and S100β, which are zinc-binding factors with altered expression in AD. The role of these proteins was also assessed in conjunction with astrocyte function and its relationship to amyloid toxicity. MT-III is a potent inhibitor of neurite outgrowth and a comprehensive examination was performed to determine its relative expression level in AD and control cases. Immunoblotting, histochemistry and analysis of mRNA levels revealed that MT-III is significantly reduced in AD tissue. Furthermore, nucleosome structure alterations suggested a non-functional MT-III promoter. Loss of normal inhibitory MT-III activity is proposed as a factor for abnormal neuritic sprouting observed in AD. Reduced MT-III may have additional consequences as demonstrated by its ability to sequester zinc and protect against the metal-induced aggregation and toxicity of the Aβ peptide. As a counterpart

to MT-III, S100β induces neurite outgrowth and is significantly elevated in AD. We have shown that S100β directly interacts with tau and that this binding is inhibited by the hyperphosphorylation associated within AD-related neurofibrillary tangles. In neuron-like cells, S100β was internalized and co-localized with intracellular tau, which was potentiated by the addition of exogenous zinc. Tau phosphorylation was unaltered but S100β uptake was accompanied by extensive neurite outgrowth. This suggests that S100β may regulate tau function and disruption of this pathway, either by changes in its expression level or metal imbalances, may contribute to the disease process. Astrocytes are key to S100β and MT-III activity and they provide support for neurons under stress conditions. The role of astrocytes was examined and we have ascertained that, with aging, these cells exhibit a diminished cytokine response and a reduced capacity to protect cells from amyloid toxicity. Similar effects are proposed to occur in aged individuals and lead to a greater susceptibility to neuronal loss. Overall these studies indicate a number of pathways involving MT-III and S100β that, along with their glial partners, can promote Alzheimer's disease-like pathological events.

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She bid me take life easy as the leaves grown on the tree
But I being young and foolish with her did not agree

"Down by the Salley gardens", WB Yeats

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SUMMARY OF ABBREVIATIONS

µCi	microCurie
µg	microgram
AD	Alzheimer's disease
$AMSO_4$	ammonium sulphate
APP	amyloid precursor protein
Aβ	amyloid-beta peptide
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CAPS	3-cyclohexylamino-l-propanesulfonic acid
CBR	Coomassie brilliant blue R-250
CPTS	copper-phthalocvanine-3,4',4",4'"-tetrasulfonic acid tetrasodium salt
DMEM	Dubelco's modified eagle medium
DNA	deoxynucleic acid
DTT	dithiothreitol
EDTA	ethylene ditetra-acetic acid
EGTA	ethylene guanyl tetra-acetic acid
FCS	fetal calf serum
GAPDH	Glyceraldehyde-3-phospate dehydrogenase
GIF	growth inhibitory factor, also called metallothionein III
HCl	hydrochloric acid
hr	hour
IgG	immunoglobulin

IL	interleukin
KCl	potassium chloride
kD	kilodalton
KLH	keyhole limpet hemocyanin
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
mL	millilitre
mM	millimolar
MN	micrococcal nuclease
mR	relative molecular weight
mRNA	messenger ribonucleic acid
MT	metallothionein
MT-1	metallothionein I
MT-2	metallothionein II
MT-3, MT-III	metallothionein III
MTT	3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide
NaCl	sodium chloride
NBT	nitroblue tetrazolium
NFT	neurofibrillary tangles
NGF	nerve growth factor
NIH	National Institutes of Health
NO	nitric oxide
PC-12	pheochromoacytoma-12 cells

PCR	polymerase chain reaction
PDTC	pyridine-2, 6-bis (thiocarboxylic) acid
PKC	posphokinase C
PMA	phorbol myrisitc acid
PMSF	phenylmethylsulfonylfluoride
PS1	presenilin 1
PS2	presenilin 2
PVDF	polyvinylidene difluoride
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SRB	sulforhodamine B
TGF	transforming growth factor
TNF	tumour necrosis factor
U	unit
Zn	zinc

PUBLICATIONS INCLUDED IN THESIS

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Abstracts

1) **Yu, WH**, and PE Fraser. S100ß interactions with tau in Alzheimer's disease and the analysis of chemical properties required. Washington: **World Alzheimer Congress.** July 8-13, 2000.

2) **Yu, WH**, Fraser PE and J McLaurin. Phenotypic and functional differences in glial cells during aging. Barcelona: **European Meeting on Glial cell Function in Health and Disease.** May 24-27, 2000.

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INTRODUCTION

1.1 Alzheimer's Disease

1.1.1 Epidemiology

Alzheimer's disease (AD) is the most common form of dementia, affecting 5% of people over the age of 65 and accounting for 2/3 of all dementia cases (Canadian Study of Health and Aging Working Group, 1994; Tomlinson, 1990; Alafuzoff *et al.*, 1987). It is predominantly a disease of the elderly, though there are some cases of early onset related to genetic factors. As the average age of the population increases, so too will the incidence rate of AD. Furthermore, improvements in other aspects of health have also decreased the mortality rate ensuring a greater portion of the population will be affected by AD. A comprehensive study on the potential impact of Alzheimer's disease in Canada has identified that in the year 2001, there will be 109,900 individuals that develop AD or some other form of dementia, with more than 2/3 (70,200) being women (Canadian Study of Health and Aging Working Group, 2000). Currently, the total number of people over 65 with AD is 238,000 (Canadian Study of Health and Aging Working Group, 1994), or approximately 0.6% of the total population. Again, over 2/3 (247,520) of this number will be women. They also forecast that by the year 2031, the number of Canadians affected by AD will exceed 500,000 (Canadian Study of Health and Aging Working Group, 1994), or approximately 1.5% of the total population.

An estimated $5.5 billion a year is spent on persons with Alzheimer's disease and related dementias in Canada (Ostbye and Crosse, 1994). Cost estimates can be further broken down into categories based on disease severity (Hux *et al.*, 1998). Based on complete

medical coverage, an individual with late stage Alzheimer's would require $36, 794 (Canadian) annually in care, whereas one at a mild to moderate stage would cost $10,000-$16,000 (Canadian) per annum. Furthermore, over 50% of all those afflicted with AD are institutionalized (Can J Aging, 1994).

Of those afflicted with Alzheimer's disease, but not living in an institution, 97% of cases require a caregiver, usually a family member, who are predominantly over 60 years of age themselves (Cdn Study of Health and Aging, 1994). Often, the primary caregivers are given little respite from their duties and consequently are also twice as likely to suffer from depression as is the general population. The financial and psychological impact of Alzheimer's disease necessitates action not only to develop strong medical support, but also expedite research initiatives.

1.1.2 Pathology of Alzheimer's disease

Originally described in 1907 by Alois Alzheimer as a clinical dementia affecting people in their forties and fifties, it has since been used to encompass a multifactorial dementia characterized by a cognitive decline with characteristic pathology (Shore and Wyatt, 1983). AD is pathologically identified by three events: presence of senile plaques, which are composed primarily of a protein called Aβ (Aβ); neurofibrillary degeneration, often into a structure called paired-helical filament, formed by hyperphosphorylated tau; and, extensive neuronal death (See Fig 1.1). While there is some evidence of inter-relationship, or cause-and-effect, between these events, their individual actions are well researched and documented.

Figure 1.1: Characteristic senile plaque (left) and neurofibrillary tangle seen in Alzheimer s disease with arrows to denote structure.

1.1.2.1 Aβ Aggregation and Senile Plaques

Aβ is formed by the cleavage of a protein called amyloid precursor protein (βAPP). Aβ has three forms of 40, 42 or 43 amino acids in length (Hardy, 1997; Selkoe, 1994; Sisodia and Price, 1995, Yankner, 1996). It is the central component in the amyloid cascade hypothesis supported by Selkoe (1994) and Yankner (1996), as well as other AD researchers. There are several reasons for the development of Aβ as the core pathology and its role in the development of AD. First, βAPP mutations produce $A\beta_{1-42}$, the more amyloidogenic form, in greater proportions relative, to $A\beta_{(1-40)}$ and are attributed to the development of familial AD (FAD) (Duff et al., 1994; Citron et al., 1992; Hardy, 1997; Selkoe, 1994; Sisodia and Price, 1995, Yankner, 1996). Second, mutant presenilins are also associated with altered processing of βAPP and result in higher amounts of $A\beta_{1-42}$ (Hardy, 1997; Selkoe, 1994; Sisodia and Price, 1995, Yankner, 1996). Third, the risk of developing Alzheimer s disease is increased in the presence of certain apoE isoforms, which has been linked to amyloidogenesis in transgenic animals and humans (Hardy, 1997; St George-Hyslop, 1998; Cruts *et al.*, 1998).

Aβ is believed to have several modes of toxicity including increasing intracellular calcium (Joseph and Han, 1992), initiating apoptosis (Cotman and Anderson, 1995), activating microglial infiltration (Meda *et al.*, 1995) and forming free radical oxygen species (Behl *et al.*, 1994; Goodman *et al.*, 1994; Klegeris and McGeer, 1997). Additional evidence supports a general phenomenon where Aβ promotes or causes the observed AD pathogenesis. *In vitro* and transgenic data provides extensive evidence of Aβ toxicity (Duff *et al.*, 1996; Chapman *et al.*, 1999; Hsiao *et al.*, 1996). It appears that Aβ must undergo fibrillization to maximize its toxicity (Mark, 1996; Pollard *et al.*, 1995; Cribbs *et al.*, 1997; Lorenzo and Yankner, 1994; Pike *et al.*, 1993). Ultimately, fibrillar Aβ aggregates into deposits that appear in the brain as plaques. In the human brain, the most apparent deposits are called senile plaques. They are composed chiefly of Aβ, but can also contain heparan sulphate glycans (HSPG) (Snow *et al.*, 1988; Kisilevsky and Snow, 1990), zinc, copper and iron (Huang *et al.*, 2000; Suh *et al.*, 2000a; Garzon-Rodriguez W, 1999; Lovell *et al.*, 1998), and microglia and cellular remnants (Sheng *et al.*, 1998; Mckenzie *et al.*, 1995; Arends *et al.*, 2000). Other components of senile plaques include complement proteins, amyloid P and apoE lipoproteins.

Aβ fibrils and senile plaques are principal hallmarks of the disease and consequently there has been intense focus on identifying therapeutic treatments aimed at preventing and removing the senile plaques. Studies performed to date to prevent fibrils and aggregation include chelation of metals which may play a role in forming Aβ fibrils, blocking Aβ-HSPG interactions, immunization against the Aβ peptide to promote its removal via the immune pathway (Schenk *et al.*, 1999; Janus *et al.*, 2000; Ard *et al.*, 1996; Shaffer *et al.*, 1995; Lee *et al.*, 1999).

1.1.2.2 Tau and Neurofibrillary Degeneration

1.1.2.2.1 Physiological functions of tau

Tau is a member of the microtubule-associated protein (MAP) family. It is responsible for the stabilization of neuronal microtubules, particularly in the development of cell processes, establishment of cell polarity and providing intracellular transport (Hutton *et al.*, 1998). Tau is found mostly in axons, as opposed to MAP2, which is largely somatodendritic. Tau is found as six isoforms of 352 to 442 amino acids in length and is encoded on one gene (Goedert *et al.*, 1997). Tau synthesis is unique as it is performed in the proximal axon upon tau mRNA translocation (Mandelkow *et al.*, 1995).

Structurally, tau contains an acidic N-terminal domain, a basic and proline-rich middle domain, a basic domain consisting of three or four internal repeats, which gives it its variable length, and a C-terminal domain. Tau is very hydrophilic, soluble and is usually in an unfolded structure. It can be phosphorylated at various sites. Some of these phosphorylation sites regulate its microtubule-binding properties. They are loosely categorized into two groups. The first consists of Ser-Pro or Thr-Pro motifs in the flanking regions of the internal repeats which are targets of proline-directed kinases such as glycogen synthase kinase 3, cyclin-dependent kinase Cdk5 or MAP kinase (Singh *et al.*, 1994; Schneider *et al.*, 1999; Baumann *et al.*, 1993; Mercken *et al.*, 1995). These sites have only a moderate influence on tau–microtubule interactions but are useful as diagnostic tools for the AD-like phosphorylation of tau. The other phosphorylation target sites include protein kinase A (e.g., Ser214), microtubule-affinity-regulating kinase (MARK; at KXGS motifs including Ser262, Ser356) or Ca^{2+}/calmodulin-dependent protein kinase (Ser416) (Schneider

et al., 1999; Zheng-Fischhofer *et al.*, 1998; Ikura *et al.*, 1998). Tau detaches from microtubules when phosphorylated at Ser262 or at Ser214, two sites associated with AD (Schneider *et al.*, 1999; Zheng-Fischhofer *et al.*, 1998).

In mitotic cells, tau is phosphorylated at several Ser-Pro motifs, including Ser214, and this results in blocking binding to microtubules (Illenberger, *et al.*, 1998). Phosphorylation of tau or related MAPs by MARKs appears to be important for the establishment of cell polarity, but hyperphosphorylation of tau by MARKs can lead to cell death (Drewes, *et al.*, 1992). Tau is essential for neurite outgrowth (Kosik and McConlogue, 1994). Tau concentration is greatest at the distal tip, and tau phosphorylation greater at the proximal end of the cell body (Black *et al.*, 1996; Mandell and Banker, 1996). Tau may not be essential for neuronal survival as suggested by compensatory MAP activity in transgenic mice lacking tau expression (Harada. *et al.*, 1994).

Tau may have other less-defined roles. As the repeat domains for tau are smaller than other MAPs, it may be a better microtubule stabilizing protein (Matus, 1994). Tau is not completely localized in axons as a portion can also be found in the plasma membrane (Brandt *et al.*, 1995). In addition, overproduction of tau may lead to its localization in dendrites (Hirokawa *et al.*, 1996). Tau has also been found in the nucleus, it can also regulate vesicular transport and anchor cellular proteins, such as phosphatase 1 (Wang *et al.*, 1993; Bulinski *et al.*, 1997; Ebneth *et al.*, 1998; 38 Liao *et al.*, 1998)

1.1.2.2.2 Pathological phosphorylation of tau and AD

In AD, tau can be altered through hyperphosphorylation at various sites (Mandelkow *et al. 1995;* Mandelkow. *et al.* 1995; Delacourte and Buee, 1997). The abnormal

phosphorylation occurs mainly at Ser-Pro and Thr-Pro sites. This event is also present in fetal tissue and mitotic cells suggesting that hyperphosphorylation of tau leads to apoptosis. As a consequence of hyperphosphorylation, tau will no longer bind microtubules, reducing the cells ability to maintain shape and disrupting intracellular communication (Mandelkow and Mandelkow, 1998). As cells respond to this event, it may produce more tau that is redirected to somatodendritic compartments. In addition, tau begins to aggregate into insoluble neurofibrillary tangles (NFT) called paired helical filaments (PHF). Structurally, PHFs are double stranded fibres of 10-20nm and cross-over repeats at 80nm on the axis of the fibre (Crowther, 1991).

Tau can also undergo ubiquination and proteolysis by proteasomes and calpains (Litersky and Johnson, 1995). While this may be a cellular attempt to reduce the amount of tau, it may also exacerbate the situation by promotion of additional hyperphosphorylation and lead to increased NFT production (Novak *et al.*, 1993). Post-aggregation glycation and cellular release of tau aggregates from dying or dead neurons is also an end consequence of hyperphosphorylation (Yan *et al.*, 1994; Vigo-Pelfrey *et al.*, 1995).

A recent tau mutation linked to frontal temporal and Parkinson dementia was found on chromosome 17 (FTDP-17) (Hutton et al., 1998). The mutation results in the development of a pathology similar to that found in Pick's disease with the characteristic hyperphosphorylation of tau and the formation of tau aggregates and filaments (Poorkaj *et al.* 1998; Spillantini *et al.*, 1998; Hutton *et al.*, 1998). The mutation, however, provides mechanistic insight into the development of neurofibrillary tangles.

1.1.2.2.3 Pathological phosphorylation of tau in other diseases

Alzheimer's disease is not the only neurodegenerative disease involving tau pathology. While most Parkinson's disease (PD) cases are associated with α-synuclein Lewy body formation, a small subset is believed to have a tauopathy based in the prefrontal region (Vermersch *et al.*, 1997). This tau pathology is similar to that found in AD. In addition, neurofibrillary degeneration has been found in cases of post-encephalitic parkinsonism (PEP) (Buee-Scherrer *et al.*, 1997; Hof *et al.*, 1992). In these cases, NFTs appear in the hippocampus, neocortex and subcortical areas (Buee-Scherrer *et al.*, 1997; Hof *et al.*, 1992).

Amyotrophic lateral sclerosis (ALS) / parkinsonism-dementia complex (PDC) is another tau-related dementia (Hirano *et al.*, 1966). Restricted to the Chamorro people of Guam, this disease is associated with widespread NFT development, but most notably in the temporal and frontal cortex, hippocampal and subcortical regions (Hirano *et al.*, 1966). In AD, NFTs are greater in neocortical layers V and VI, whereas ALS/PDC appears in layers II and III (Hof *et al.*, 1992; Hof *et al.*, 1991).

Progressive supranuclear palsy (PSP) is a late-onset atypical parkinsonian disorder originally characterized in 1964 (Steele *et al.*, 1964). PSP neuropathology includes neuronal loss, gliosis and NFT formation of the basal ganglia, brainstem and cerebellum (Steele *et al.*, 1964). Structurally, NFTs are different between AD and PSP, as PHFs are found in AD (Kidd, 1963) and straight filaments are present in PSP (Tellez-Nagel and Wisniewski, 1973; Tomonaga, 1977). In addition, while a triplet of tau bands are found in AD (55, 64 and 69kD) there is only a doublet (64 and 69kD) in PSP (Flament *et al.*, 1991). An additional minor band can be found at 74kD (Mailliot *et al.*, 1998). NFTs in PSP are found in the

subcortex, but spread to the neocortex with the progression of the disease (Vermersch *et al.*, 1994).

Corticobasal degeneration (CBD) is another dementia that has tau pathology. This rare motor degenerative condition is associated with frontoparietal atrophy (Litvan *et al.*, 1996). This disease is characterized by the presence of astrocytic plaques and tau-positive inclusions in the white matter (Feany and Dickson, 1995, Feany *et al.*, 1996). As with PSP, tau is found as a 64 and 69kD doublet, though they may be constitutively different (Ksiezak-Reding *et al.*, 1994)

Another group of tauopathies is based in the frontotemporal lobe. There are two forms, Pick's disease and non-AD, non-Pick's frontal lobe degeneration. Pick's disease is a rare neurodegenerative disorder characterized by mood disturbances and progressive language impoverishment leading to mutism (Constantinidis *et al.*, 1974). Neuropathologically, Pick's disease displays extensive frontotemporal lobar atrophy, gliosis, neuronal loss or distension and presence neuronal inclusions called Pick bodies in both cortical and subcortical regions (Brion *et al.*, 1991; Tissot and Constantinidis, 1985). Pick bodies are PHF-tau immunopositive, with a higher density in hippocampus than in the neocortex (Buee-Scherrer *et al.*, 1996; Delacourte *et al.*, 1996; Hof *et al.*, 1994). In the hippocampus, Pick bodies are found in the dentate gyrus in the CA1 field, the subiculum and the entorhinal cortex, whereas the neocortex, they are mainly found in layers II and VI the anterior segment of temporal and frontal lobes. Some NFTs can also be found in the hippocampus and are usually considered as part of aging. Prominent 55 and 64kD tau doublets are seen by Western analysis (Buee-Scherrer *et al.*, 1996; Delacourte *et al.*, 1996), as well as a minor 69kD band.

Non-Alzheimer non-Pick frontal lobe degeneration is more common. Clinically, the frontal cortex hallmarks are like Pick's disease (neuronal loss and gliosis), but neuropathologically, there does not appear to be any tau phosphorylation (Brun *et al.*, 1994; Buee-Scherrer *et al.*, 1996).

Recently, genetic mutations have been implicated in tau-related dementias. Familial frontotemporal dementias (FTD) have been linked to chromosome 17. In 1994, Wilhelmsen and colleagues described an autosomal dominantly-inherited disease familial FTD, characterized by adult-onset behavioral disturbances, frontal lobe dementia, parkinsonism and amyotrophy. They identified a genetic linkage between the pathology seen in disinhibition–dementia–parkinsonism–amyotrophy complex (DDPAC) and chromosome 17q21-22, which encodes tau (Lynch *et al.*, 1994; Wilhelmsen *et al.*, 1994). Since then, other families have also been found (Hutton *et al.*, 1997; Bird *et al.*, 1997; Heutink *et al.*, 1997; Murrell *et al.*, 1997; Wijker *et al.*, 1996). Collectively, they have been classified as frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (Foster *et al.*, 1997). Neuropathologically, FTD brains are atrophied in the frontal and temporal cortex, with extensive neuronal cell loss, grey and white matter gliosis and spongiosis. Common to the pathology is the filamentous aberrations in neuronal and glial cells. They also lack Aß aggregates (Foster *et al.*, 1997; Spillantini *et al.*, 1998).

The FTD tau mutations come in two forms: i) mutations affecting the alternative splicing of exon 10 lead to alterations in the 4R:3R-tau ratio; and ii) mutations that modify tau interactions with microtubules. 4R tau resembles the 64/69kD doublet seen in PSP and CBD and can exhibit a twisted ribbon filament in neurons and glia. The other form results in reduced tau binding to microtubules and destabilize microtubules. The tau pathology is

similar to AD in that PHFs and straight filaments are found. PSP polymorphisms are autosomal dominant mutations. Unlike FTD, they are caused by an intron 9 dinucleotide repeat (Conrad *et al.*, 1997). The polymorphism was also found in other pathologies including CBD and Parkinson's disease (DiMaria *et al.*, 2000; Pastor *et al.*, 2000). Myotonic dystrophy is another autosomal dominant and is characterized by a highly variable progressive multisystemic disorder characterized principally by myotonia, muscular atrophy, cataract and endocrine dysfunction (Harper, 1989), with some impairment of cognitive. The disease is due to a defect in the CTG trinucleotide repeat in the untranslated region of a gene (Buxton *et al.*, 1992). This encodes the Ser / Thr protein kinase (myotonic dystrophy protein kinase) (DMPK) and is found on chromosome 19 (Buxton *et al.*, 1992). Myotonic dystrophy results in reduced brain weight and gyral abnormalities. Histologically, there is a disordered cortical arrangement with neurons present in subcortical white matter, as well as cytoplasmic inclusion bodies (Ono *et al.*, 1987). NFT can also be present, though not as frequent, usually in the temporal lobe (Kiuchi *et al.*, 1991). By Western analysis, it is possible to detect PHF-tau in the hippocampus, entorhinal and temporal cortices. The tau found is mostly of the 55kD variety and is usually devoid of the 69kD isoform (Vermersch *et al.*, 1996). The presentation of NFTs usually coincides with the presence of the Ser / Thr protein (Vermersch et al., 1996).

Other diseases that display NFTs are prion diseases and Down's syndrome. Gerstmann–Straussler–Scheinker disease (GSS) is an autosomal dominant neurodegenerative disorder with resulting in ataxia, spastic paraparesis, extrapyramidal signs, and dementia (Gerstmann *et al.*, 1936). The predominant neuropathology is the presence of widespread prion plaques, severe neuronal loss and spongiosis. NFTs similar to PHF-tau of AD may

also be present (Ghetti *et al.*, 1995; Ghetti *et al.*, 1996). The immunoreactive tau is the same as that seen in AD, or a degradative product (Tranchant *et al.*, 1997).

Down syndrome (DS) patients have numerous neurological and developmental dysfunctions. DS usually results in the early onset of dementia (before 50 years of age) (Hof *et al.*, 1999). Neuropathologically, there is severe neuronal loss of the hippocampus, neocortex and subcortical areas. Aß deposits occur prior to neuronal loss, as do NFTs. Diffuse Aß deposits are seen within 15 years, followed by massive deposition of senile plaques in the next decade of life. This is followed by neurofibrillary (Hof *et al.*, 1995a; Mailliot *et al.*, 1989). The distribution of NFTs and amyloid plaques is similar to that seen in AD (Hof *et al.*, 1995a). While DS Aß deposits may be a result of a triplication of the APP gene, the presence of NFTs may indicate a neuropathological link between tau and Aß.

1.1.2.3 Neuronal Death

While the development of Aβ aggregates and senile plaques, as well as the presence of neurofibrillary degeneration can cause neuronal death, other factors are also attributed to a neuron's demise. Excitoxicity, metal induction, excess cytokine and inflammatory responses and trauma may further the development of AD. Neuronal death, which occurs in Alzheimer's disease, is a multi-factorial process and it is critical to identify the event sequences that may promote the pathology.

Several investigations have centred on the production of free radicals. The homeostasis between free radical generation and removal is usually maintained by catalase and glutathione peroxidase which remove the noxious H_2O_2. An excess production of reactive oxygen species can lead to several downstream events including formation of

glycation end products (Smith *et al.*, 1994; Ledesma *et al.*, 1994; Yan *et al.*, 1994); nitration (Good *et al.*, 1996); lipid peroxidation adduction products (Montine *et al.*, 1996); and, production of carbonyl-modified neurofilament protein and free carbonyls (Smith *et al.*, 1996; Smith *et al.*, 1991). These events occur in AD but are not necessarily pathogenic, though they often leave neurons vulnerable to additional toxic insults.

In AD, there are several potential sources of free radical generators such as iron and copper in Aβ deposits (Good *et al.*, 1992; Smith *et al.*, 1997). Iron is a potent catalyzer of OH^- from H2O2. It can also aid in the formation of advanced glycation end products (Smith *et al.*, 1994). The increase in the accumulation of iron and copper is believed to be the major source reactive oxygen species production. This in turn is responsible not only for the numerous oxidative stress markers that appear within NFT and senile plaques, but also for the more global oxidative stress parameters observed in AD. Aluminum, which has also been found in NFTs (Good *et al.*, 1992) can further stimulate iron-induced lipid peroxidation (Oteiza, 1994). Activated microglia which are mobilized in AD (Cras *et al.*, 1990) are an abundant source of NO and O_2^- (Colton and Gilbert, 1987). Aβ has also been directly implicated in forming reactive oxygen species (Hensley *et al.*, 1994; Sayre *et al.*, 1997). Advanced glycation end products in the presence of transition metals can undergo redox cycling and also produce reactive oxygen species (Yan *et al.*, 1994; Yan *et al.*, 1995). Furthermore, advanced glycation end products and Aβ activate specific receptors, such as the receptor for advanced glycation end products (RAGE) and class A scavenger-receptor that can increase reactive oxygen production (Yan *et al.*, 1996; El Khoury *et al.*, 1996). Mitochondrial mutations (Corral-Debrinski *et al.*, 1994; Davis *et al.*, 1997) or deficiencies in

key metabolic enzymes (Sorbi *et al.*, 1983; Parker *et al.*, 1990; Blass *et al.*, 1990; Sheu *et al.*, 1985) can also alter the homeostasis of free radical levels.

Neuronal death via oxidative reactions can occur globally, but it is the selective loss of neurons, particularly in the limbic region encompassing the hippocampal, entorhinal and subcortical nuclear regions that typifies Alzheimer's disease. (Hyman and Gomez-Isla, 1994). One possibility for neuronal susceptibility is that dystrophic axons or neurites, particularly those of larger lengths make them more susceptible to damage (Lewis *et al.*, 1987; Braak *et al.*, 1994). Myelinated, short axons appear less vulnerable to damage in cortical and subcortical neurons (McGeer *et al.*, 1990; Braak and Braak 1996). In AD, the presence of dystrophic neurites may result from overproduction of tau, as well as a compensatory mechanism to reestablish neural connections lost when other neurons die. A consequence of neuritic outgrowth, however, is the increased exposure to toxic insults and ultimately, increased neuronal death (Uchida *et al.*, 1988; Griffin *et al.*, 1989).

1.1.3 Other Factors Contributing to the Development Alzheimer's disease.

Other events and modulating factors are also involved in the pathogenic development of Alzheimer's disease. Within the reduction in the neuronal population, there is a marked decline in the cholinergic-producing neuronal population (Mayeux, 1990; MacDermott *et al.*, 1978). There is also an increase in monoamine oxidase levels, potentially leading to a decrease in the monoamine neurotransmitter population (Danielczyk *et al.*, 1988; Murphy, 1978; Deary and Whalley, 1988).

Unlike neurons, astrocytes and microglia are increased over the duration of the disease's progression beyond the normal increases observed in the aging brain (Murphy, Jr.,

et al., 2000; Halliday *et al.*, 2000). Reactive astrocytes and microglia produce proteins that are responsible for the complement system in the brain (Emmerling *et al.*, 2000; Eikelenboom *et al.*, 2000; Halliday *et al.*, 2000). The cytokines produced have been implicated in the modulation of AD development, and sometimes, as in the case of interleukin 1β (IL-1β) may precede any pathology (Li *et al.*, 2000; Sheng *et al.*, 1998; Griffin *et al.*, 1998). Reactive astrocytes are known to extend their processes into the senile plaque cores, presumably to reduce the Aβ load (Akiyama *et al.*, 2000). Futhermore, Aβ upregulates astrocytic cytokines such as IL-1β, interleukin 6 (IL-6), S100β and tumour necrosis factor-α (TNFα) (Sheng *et al.*, 1998; Arends *et al.*, 2000; Sheng *et al.*, 1995). Microglia and astrocytes provide an important role in the nervous system through its removal of Aβ and dead cells (Eikelenboom and Veerhuis, 1996; Sasaki *et al.*, 1997; Kopec and Carroll, 1998). They also produce cytokines that are involved in a positive feedback loop involving IL-1β being induced by Aβ and in turn, promoting the amount of Aβ produced (Barger and Harmon, 1997; Griffin *et al.*, 1998).

In addition to complement factors that are upregulated in Alzheimer's disease, neurotrophic factors are also altered in the disease process. Some proteins like BDNF are reduced, whereas others like TGF-β and S100β are increased (Leszek and Gasioroski, 1994; Van Eldik and Griffin, 1994; Tan *et al.*, 1999). In addition, Alzheimer's brains have a higher neurotrophic level of activity than control brains (Uchida *et al.*, 1988). The imbalance of neurotrophic factors is believed to contribute to the pathogenesis through the development of dystrophic neurites (Marx *et al.*, 1999; Dickson, 1999; Marzolo *et al.*, 2000). Dystrophic neurites may be a result of increased tau production (Sheng *et al.*, 2000), compensation to decreased neuronal population or response to the presence of Aβ plaques (Matsumoto *et al.*,

2000; Sasaki *et al.*, 1996; Durany *et al.*, 1999). A common element in these events is that neuronal survival and regulation are disrupted in Alzheimer's disease. Furthermore, compensatory mechanisms appear to exacerbate the process and accelerate the pathogenesis.

1.1.4 Metal in Alzheimer's disease

Metals have been implicated in the Alzheimer disease pathogenesis. Aluminum, iron, copper and zinc have been investigated due to their presence within senile plaques and neurofibrillary tangles. Aluminum has been implicated as a modulating factor in Alzheimer's disease because it is known to alter enzymes such as monoamine oxidase B, binds chromatin and is found in the neurofibrillary tangles (Zatta *et al.*, 1999; Murayama *et al.*, 1999; Savory *et al.*, 1998; Jones *et al.*, 1998; Reusche, 1997; Shin *et al.*, 1994; McLachlan *et al.*, 1991). Epidemiological evidence has implicated aluminum, used to treat municipal drinking water, as a risk factor in the development of the disease (Rondeau *et al.*, 2000; Martyn *et al.*, 1997; Forbes and Agwani, 1994; Corrigan *et al.*, 1993; Murray *et al.*, 1991).

Iron is another metal implicated in Alzheimer's disease. In the AD brain there is a higher level of free or loosely associated ferric iron, particularly in the hippocampal region. Ferric iron can also be liberated from plaques (Shimohama *et al.*, 2000; Garlind *et al.*, 1998; Davies *et al.*, 1997). Finally, iron is a potent free radical agent and oxidation is another phenomenon of AD (Castellani, *et al.*, 1999; Dedman *et al.*, 1992).

Calcium has also been extensively examined and has been shown to be selectively increased in AD (Boissiere *et al.*, 1996; Hayashi, 1996). Calcium is implicated in reactive oxidative stress and excitotoxicity, possibly through dysregulation of calcium homeostasis

(Cyr *et al.*, 2000; Dore *et al.*, 2000; Zazpe and Del Rio, 1997; Raidoo and Bhoola, 1998; Boissiere *et al.*, 1996; Hayashi, 1996). Calcium is known to interact with several proteins such as APP, calsenilin and phosphate kinase C (PKC) leading to hyperphosphorylation of tau (Cyr *et al.*, 2000; Dore *et al.*, 2000; Zazpe and Del Rio, 1997; Raidoo and Bhoola, 1998; Yamada *et al.*, 1997; Boissiere *et al.*, 1996; Hayashi, 1996; Buxbaum, *et al.*, 1998)

Currently, zinc and copper have become important metal candidates in AD pathogenesis. Zinc is increased in the AD brain possibly via entry through glutamate receptors and can act as an excitotoxic agent. Furthermore, zinc and copper have proven to be potent aggregators of Aβ (Yang *et al.*, 2000; Castellani *et al.*, 1999; Huang *et al.*, 2000; Cuajungco *et al.*, 2000; Garzon-Rodriguez *et al.*, 1999; Liu *et al.*, 1999; Chaney *et al.*, 1998; Huang *et al.*, 1997; Bush *et al.*, 1994; Bush *et al.*, 1993). Further evidence of a role for zinc and copper in AD is the presence of these metals in senile plaques (Suh *et al.*, 2000a). Zinc is a tightly regulated metal, predominantly stored in metal binding proteins such as metallothioneins. In addition, it is essential for the function of over 300 enzymes (Pasinelli *et al.*, 2000; Coulter, 2000; Nolo *et al.*, 2000; Dineley *et al.*, 2000; Franco-Pons *et al.*, 2000; Jo *et al.*, 2000; Suh *et al.*, 2000b; Suh *et al.*, 2000c; Sensi *et al.*, 1999; Sensi *et al.*, 1997; Ross *et al.*, 1997; Aschner *et al.*, 1997; Frederickson and Moncrieff, 1994). In AD, it is postulated that there is a dysregulation in zinc function and turnover, with the net effect that zinc activity promotes some of the pathological events in Alzheimer's disease (Suh *et al.*, 1999; Kim *et al.*, 1999a; Kim *et al.*, 1999b; Koh and Choi, 1994; Mesco *et al.*, 1991; Lees *et al.*, 1990).

1.1.5 The Genetics of Alzheimer's Disease

In the past decade, genetic mapping of familial Alzheimer's disease has led to the discovery of several genes that predispose an individual to the disease. Currently, it is believed that between 5-15% of all Alzheimer's cases are caused by genetic mutations. The remaining cases are a combination of unknown genetic predispositions and environmental factors.

There are currently four genes identified with the onset of Alzheimer's disease. Three of the four genes, βAPP, PS1 and PS2, are considered early onset instigators, whereas the fourth, APOE, is considered a modulator of the disease process enhancing the risk of the development of the disease. Other candidate genes such as BACE, IDE, α-2 macroglobulin (A2M) and nicastrin have also been implicated, but not proven to directly cause AD through genetic mutation.

1.1.5.1 The β-Amyloid Precursor Protein Gene

The first gene to be identified was the β-amyloid precursor protein (βAPP) gene located on chromosome 21 and encoding a protein composed of up to 770 amino acids (Kornberg *et al.*, 1989; Kang *et al.*, 1987; Goldgaber *et al.*, 1987; Robakis *et al.*, 1988; Tanzi *et al.*, 1987; Selkoe, 1994). There are three proteolytic sites identified which involve the processing of Aβ. The first is an α-secretase cleavage within the Aβ peptide sequence that releases the extracellular N-terminus of βAPP. The other pathway involves proteolysis via β- and γ- secretases and generates Aβ peptides of 40-43 amino acids. The longer, and potentially more neurotoxic isoforms, $A\beta_{42-43}$, are elevated in the brains of individuals affected with AD suggesting a shared Aβ pathogenic mechanism.

β-secretase activity is believed to be performed by an aspartyl protease, named β-site APP-cleaving enzyme (BACE) (Vassar *et al.*, 1999; Yan *et al.*, 1999; Sinha *et al.*, 1999; Hussain *et al.*, 1999; Lin *et al.*, 2000). BACE overexpression in cultured cells increased Aβ secretion with a concomitant reduction in the α-secretase product, whereas, inhibition of BACE expression lowered production of Aβ peptides. BACE is produced from Pro-BACE, a type I transmembrane protein that undergoes a furin-like cleavage in the endoplasmic reticulum and glycosylation en route to the cell surface (Bennett *et al.*, 2000a; Huse *et al.*, 2000; Haniu *et al.*, 2000; Capell *et al.*, 2000a).

The amyloid cascade hypothesis proposed by Selkoe (1994) and Yankner (1996) suggests that aberrant production and aggregation of the Aβ peptides (especially $A\beta_{42-43}$) is central to AD neuropathology (Selkoe, 1994; Yankner, 1996). The common pathological effect of all four AD-linked genes is to alter βAPP processing and promote Aβ deposition as extracellular plaques. Pathogenic mutations of the βAPP gene are clustered near the α-, β-, or γ-secretase cleavage sites for amino acids 670/671, 692, 716, and 717, suggesting direct consequences on βAPP cleavage and potential promotion of the Aβ formation pathway (Mullan *et al.*, 1992). For example, an elevation of Aβ peptide level is associated with mutations at codons 716 and 717 (Citron *et al.*, 1992; Shoji *et al.*, 1992; Suzuki *et al.*, 1994; Haass *et al.*, 1994; Haass *et al.*, 1995; Eckman *et al.*, 1997), while Lys_{670}⇒Asn and Met_{671}⇒Leu double mutations increase the amount of Aβ produced (Citron *et al.*, 1994). Two other mutations, Val715Met and Glu693Gly, reduce total Aβ production (Ancolio *et al.*, 1999; Nilsberth *et al.*, 2000). In contrast, the Ala_{692}⇒Gly mutation reduces α-secretase cleavage but increases the heterogeneity of secreted Aβ species (Haass *et al.*, 1994).

The ratio of $A\beta_{42}$ to overall Aβ species may be a more reliable indicator for AD pathology than absolute levels of $A\beta_{42}$ or total Aβ. A recent study reported increased $A\beta_{40}$ and $A\beta_{42}$ levels, in advance of plaque formation, in postmortem AD brains that correlated with the severity of dementia (Naslund *et al.*, 2000). These results suggest that Aβ protofibril formation may initiate AD pathogenesis and that therapeutics targeting Aβ peptides early in the course of the disease may be effective.

Currently, immunizing transgenic mice expressing human mutant βAPP with $A\beta_{42}$ peptide has resulted in a reduction in Aβ plaque load (Schenk *et al.*, 1999). The immunized animals subsequently developed fewer plaques raising the possibility of an AD vaccine. A second study independently reported that immunized mice showed improved performance on behavioral tasks (Janus *et al.*, 2000). Interestingly, the functional cognitive recovery was detected prior to the appearance of plaques, offering some utility as a preventative measure. Clinical trials are underway to determine the effectiveness of this strategy in humans.

1.1.5.2 Presenilins

Mutations in the presenilin (PS) genes (PS1 and PS2) account for a majority of the early-onset FAD cases (Sherrington *et al.*, 1995; Rogaev *et al.*, 1995; Levy-Lahad *et al.*, 1995). PS1 and PS2 are conserved proteins with a molecular weight of 50kDa and 7-10 putative transmembrane domains. Cleavage of presenilin within the cytoplasmic loop domain yields N- and C- terminal fragments of approximately 35 kDa and 18-20 kDa, respectively (Thinakaran *et al.*, 1996; Podlisny *et al.*, 1997). This endoproteolysis is tightly regulated and readily saturated though the two fragments remain associated following cleavage in a multimeric complex (Thinakaran *et al.*, 1998; Saura *et al.*, 1999). The PS1

holoprotein and its endoproteolytic fragments can be isolated in distinct high molecular weight complex with a molecular range of 180-1000 kDa suggesting several multi-protein configurations (Capell *et al.*, 1998; Thinakaran *et al.*, 1998; Yu *et al.*, 1998; Lee *et al.*, 1998; Annaert *et al.*, 1999; Li *et al.*, 2000a; Yu *et al.*, 2000a). Another proteolytic mechanism, possibly associated with apoptosis, involves members of the caspase 3 family of proteases (Kim *et al.*, 1997a; Grunberg *et al.*, 1998; Brockhaus *et al.*, 1998). Similar, but independent, protein complexes can also be formed by presenilin-2.

Presenilins regulation within these complexes is believed to be related to its function as free presenilin molecules are subject to rapid proteosome mediated degradation (Fraser *et al.*, 1998). Candidate presenilin interacting proteins that have been reported include filamin (Zhang *et al.*, 1998), calsenilin (Buxbaum *et al.*, 1998), rab11 (Dumanchin *et al.*, 1999), notch (Ray *et al.*, 1999), QM/Jif-1 (Imafuku *et al.*, 1999), Bcl-2 (Alberici *et al.*, 1999), Bcl-X(L) (Passer *et al.*, 1999), E-cadherin (Georgakopoulos *et al.*, 1999) and GDI (Scheper *et al.*, 2000). Their activity in the complex, however, has not been elucidated. The most common interactions are with members of the armadillo protein family including β- and δ-catenin, neural plakophilin-related armadillo protein (NPRAP), and p0071 (Zhou *et al.*, 1997; Yu *et al.*, 1998; Levesque *et al.*, 1999; Stahl *et al.*, 1999). There have been inconsistent reports of a direct interaction between PS1, individually or within the protein complex, and βAPP (Weidemann *et al.*, 1997; Xia *et al.*, 1997; Kim *et al.*, 1997b; Thinakaran *et al.*, 1998).

Nicastrin is a recently identified constituent of the PS1and PS2 high molecular weight complexes. It is also a type I transmembrane protein with a molecular weight ranging from 75 kDa for the native protein to 120 kDa when glycoslyated (Yu *et al.*, 2000a). Nicastrin activity is critical for the function of PS based on several pieces of evidence: (i) a component

of PS complexes binds both PS1 and βAPP; (ii) mutations engineered within the conserved DYIGs domain of nicastrin increase $A\beta_{40}$ and $A\beta_{42}$ secretion; (iii) deletion of the DYIGs region reduce Aβ production; and, (iv) inhibition of nicastrin expression in *Caenorhabditis elegans* (*C. elegans*) reproduces the PS homologue null mutation phenotype.

Presenilins share significant homology with *C. elegans* proteins SEL-12 and HOP-1 which are components of the Notch signaling pathway (Levitan and Greenwald, 1995; Li and Greenwald, 1997; Westlund *et al.*, 1999; Wittenburg *et al.*, 2000). It is also functionally conserved as suggested by the rescue of sel-12 mutants by human PS1 (Levitan *et al.*, 1996; Baumeister *et al.*, 1997). The common phenotype of $PS1^{-/-}$ mice and $Notch^{-/-}$ mice supports a role of PS1 in mammalian Notch signaling (Conlon *et al.*, 1995; Wong *et al.*, 1997; Shen *et al.*, 1997). Furthermore, PS1 is essential for the intramembranous proteolytic cleavage of the Notch receptor (De Strooper *et al.*, 1999; Struhl and Greenwald, 1999; Song *et al.*, 1999), with a report of the physical interaction between PS1 and the Notch1 receptor from human and *drosophila* (Ray *et al.*, 1999). Notch processing may account for at least part of the developmental phenotype of PS1 deficient mice, its relevance to AD pathogenesis is probably minimal, since Notch localization and proteolysis is not greatly affected in cells expressing AD-associated PS1 mutants (Struhl and Greenwald, 1999; Ye *et al.*, 1999).

Multiple lines of evidence implicate presenilin as the γ-secretase for βAPP processing. First, presenilin mutations can increase the production of $A\beta_{42-43}$ peptides by altering the γ-secretase cleavage (Scheuner *et al.*, 1996; Borchelt *et al.*, 1996; Duff *et al.*, 1996; Citron *et al.*, 1997). Second, PS1-deficient mice lack γ-secretase activity and consequently accumulate intracellular C-terminal stubs of βAPP but have a reduction in Aβ secretion (De Strooper *et al.*, 1998). Third, mutation of two conserved transmembrane

aspartate residues (Asp257 and Asp385) of PS1 inactivate γ-secretase activity and reduce Aβ secretion (Wolfe *et al.*, 1999; Steiner *et al.*, 1999). Finally, photoactivated inhibitors of γ-secretase also label PS1 and PS2 (Li *et al.*, 2000b). The latter finding has implicated presenilin as part of or the primary constituent of the γ-secretase active site.

Other studies, however, suggest presenilin control of γ-secretase activity is indirect, possibly by regulating βAPP or Notch trafficking, or activation of γ-secretase. First, expression of a naturally occurring splice variant of PS1 with a deletion of the exon 8 region which contains the essential Asp257 residue failed to reduce Aβ production but prevented Notch cleavage (Capell *et al.*, 2000b). Second, substitution of charged residues at familial Alzheimer's disease (FAD)-associated mutation sites L_{286}→V, to L_{286}→E or L_{286}→R, doubled the $A\beta_{40-42}$ production, but inhibited Notch cleavage (Kulic *et al.*, 2000). Third, a mutation of a conserved cysteine in C. elegans sel12 (Cys_{60}→Ser) impairs Notch processing, whereas the corresponding mammalian PS1 mutation (Cys_{92}→Ser) increased $A\beta_{42}$ secretion (Zhang *et al.*, 2000). These three studies suggest that the roles of the presenilins in the two proteolytic activities are related but functionally distinct. Finally, the inability of PS1 mutants lacking the conserved aspartyl residues to form normal high molecular weight complexes also argues that the mutant PS1 may not be structurally sound (Yu *et al.*, 2000b).

Approximately 50% of early onset FAD cases are associated with PS1 mutations. Currently there are more than 80 different missense mutations and two splicing defects reported in the PS1 gene (Tandon *et al.*, 2000; Rogaeva *et al.*, 2000). Most of these mutations are within the highly conserved transmembrane domains, or near putative membrane interfaces, or within the N-terminal hydrophobic or C-terminal hydrophobic residues of the putative TM6-TM7 loop domain (Cruts *et al.*, 1998). All PS1 mutations are associated with

presenile AD, except the Glu_{318}⇒Gly substitution, a rare polymorphism that is unrelated to AD (Mattila *et al.*, 1998). Postmortem analysis of patients with PS1-linked FAD show marked accumulation of longer Aβ isoforms relative to brains from unaffected individuals or to subjects with sporadic AD (Tamaoka *et al.*, 1998). This supports recent work showing an enhancement of $A\beta_{42}$ secretion by different PS1 mutations (Murayama *et al.*, 1999). Additional data suggests that PS1 and PS2 mutations modulate cellular sensitivity to apoptosis induced by a variety of factors (Vito *et al.*, 1996; Wolozin *et al.*, 1996; Guo *et al.*, 1997; Deng *et al.*, 1996). PS2 mutations are less common and have a variable phenotype (Sherrington *et al.*, 1996; Bird *et al.*, 1996). Since PS2 mutations can also increase the secretion of long-tailed Aβ peptides it is likely that PS1 and PS2 have overlapping modes of activity (Scheuner *et al.*, 1996; Citron *et al.*, 1997).

1.1.5.3 Apolipoprotein E (APOE)

Another genetic locus for inherited susceptibility to AD is the Apolipoprotein E (APOE) gene on chromosome 19, which expresses a lipoprotein involved in cholesterol metabolism (Pericak-Vance *et al.*, 1991; Strittmatter *et al.*, 1993a). There are three common isoforms of the APOE gene that encodes alleles ε2, ε3 and ε4. The ε4 polymorphism is significantly over-represented in AD subjects, 40% compared to 15% in the general population (Saunders *et al.*, 1993), whereas the frequency of the ε2 allele is reduced from 10% (general population) to 2% in AD (Corder *et al.*, 1994). The mean age of onset of AD is less than 70 years among the ε4/ε4 population, but over 90 years for the ε2/ε3 population (Roses, 1998). The link between the ε4 allele and AD has been confirmed in several studies across multiple ethnic groups, with conflicting results detected in studies in black Americans

and Hispanics (Maestre *et al.*, 1995; Hendrie *et al.*, 1995; Tang *et al.*, 1998). Genotyping for the ε4 allele as the sole criterion to diagnose AD is ambiguous, but is useful when in combination with other clinical measures (Mayeux *et al.*, 1998).

Several studies indicate that βAPP processing is modulated by ε4/ε2 polymorphisms. Direct interaction between APOE products and metabolites of βAPP is evident *in vitro*, where ε4 binds Aβ more avidly than ε3 (Strittmatter *et al.*, 1993a; Strittmatter *et al.*, 1993b). Both APOE and Aβ compete for clearance through the low density lipoprotein receptor-related protein (LRP) (Kounnas *et al.*, 1995). The presence of one or two ε4 alleles was found to have a positive correlation with the relative density of Aβ plaques (Schmechel *et al.*, 1993). Overexpression of human βAPP with the Val_{717}→Phe mutation promotes the development of extensive extracellular deposits of Aβ in transgenic mice which carry an intact endogenous APOE gene, but not in mice with an $APOE^{-/-}$ background (Bales *et al.*, 1997). There is also a possible link between APOE and neurofibrillary tangles as suggested by binding experiments which show strong interaction of tau or MAP2 with ε3, but not ε4, potentially implicating ε4 in the generation of hyperphosphorylated tau (Strittmatter *et al.*, 1994; Huang *et al.*, 1994; Huang *et al.*, 1995). Finally, studies have provided evidence for APOE involvement in the reorganization of neurites following neuronal injury, and suggest differential regulation of repair mechanisms by the isoforms (Masliah *et al.*, 1995; Nathan *et al.*, 1994; Nathan *et al.*, 1995). Though APOE allele selection may not be sufficient to cause AD it appears to provoke neuronal degeneration by preventing normal repair mechanisms following neuronal damage.

1.1.5.4 Other Linkage Analysis Studies

Mutations in three known genes (PS1, PS2 and APP) are responsible for 18-50% of all autosomal dominant AD cases, implying that the cause of the disease remains unexplained in at least half of the early-onset cases. In addition, more than 40% of cases with late-onset AD cannot be attributed to the presence of the APOE ε4 allele. To date, two complete genome screens have been published for late-onset AD (Pericak-Vance *et al.*, 1991; Kehoe *et al.*, 1999). The highest LOD scores were obtained for chromosomes 12, 1, 9, 10, and 19, the last one representing the APOE locus. There are 20 peaks with multipoint nonparametric scores greater than one, though many are likely false positive results and must have to be confirmed in independent data sets. A subsequent study confirmed the presence of an Alzheimer's susceptibility gene on chromosome 12 (Rogaeva *et al.*, 1998). The strongest evidence for linkage, however, was obtained at the polymorphic markers D12S96 and D12S358, which differs from the original finding around D12S1057. The conflicting results may be due to genetic and neuropathological heterogeneity of Alzheimer's disease. Mixed pathological phenotypes have resulted from linkage analysis as the mapping of the chromosome 12 locus in the original data set revealed significant association at marker D12S1075 based on families presenting with Lewy bodies. Furthermore, a recent report on an unusual form of FAD without tau pathology provided evidence for linkage with chromosome 3 markers (Poduslo *et al.*, 1999).

The specific multigenic nature of complex traits is not usually a factor in the statistical analysis. It is only recently that methods of multigene analysis have begun to be proposed (Cordell *et al.*, 2000). This approach together with a new generation of genetic

markers, such as single-nucleotide polymorphisms (SNPs) (Irizarry *et al.*, 2000) will potentially lead to the discovery of new AD genes.

1.1.5.5 Association Studies

In recent years, many candidate genes have been reported to be associated to Alzheimer's disease though they have been difficult to replicate. The complex nature of AD, especially the late-onset form, confounds the application of existing analytical techniques. Some analyses have employed family-based association methods and are theoretically better matched for genetic background than simple allelic association studies (Terwilliger and Ott, 1992; Spielman and Ewens, 1996). This approach was used to identify an intronic 5 bp-deletion polymorphism of α2-macroglobulin (A2M) gene, which is located on chromosome 12 (Jones *et al.*, 1992). A2M is present in senile plaques and may play a role in the metabolism of Aβ peptide. This association, however, has not been confirmed. Nine out of eleven reports revealed no association of A2M deletion allele with AD (Wavrant-DeVrieze *et al.*, 1999; Kovacs *et al.*, 1999; Crawford *et al.*, 1999; Korovaitseva *et al.*, 1999; Shibata *et al.*, 1999; Chen *et al.*, 1999; Hu *et al.*, 1999; Singleton *et al.*, 1999; Myllykangas *et al.*, 1999). In addition, studies on the functional significance of this polymorphism failed to demonstrate its biological effect (Carter *et al.*, 1992; Gibson *et al.*, 2000). Furthermore, another polymorphism at the A2M gene (I1000V) also failed as a potential susceptibility marker for late-onset AD (Koster *et al.*, 2000; Shibata *et al.*, 2000). Currently, many scientists believe that the potential power of population stratification may have been overestimated and that family-based control data are less efficient for detection of linkage disequilibrium than are case control studies (Morton and Collins, 1998).

Case-control allelic association tests have yielded positive results on a number of genes, many of which are plausible biochemical candidate genes: cystatin C (Crawford *et al.*, 2000), α1-chymotrypsin (Kamboh *et al.*, 1995), very low density lipoprotein receptor (Okuizumi *et al.*, 1995), LRP (Kang *et al.*, 1997), K-variant of butyrylcholinesterase (Lehmann *et al.*, 1997), bleomycin hydroxylase (Montoya *et al.*, 1998), N-acetyl transferase (Rocha *et al.*, 1999), FE65 protein (Hu *et al.*, 1998) and endothelial nitric oxide synthase (Dahiyat *et al.*, 1999). In addition, transcription regulation of the four known AD genes might be an important player in the neurodegenerative process (Theuns and Van Broeckhoven, 2000). In agreement with this, a recent association study proposed that a genetic variation in the transcriptional factor LBP-1c/CP2/LSF gene on chromosome 12 is a genetic determinant of AD (Lambert *et al.*, 2000). Unfortunately, most of these findings have not been as consistent as the association found between AD and the ε4 allele of APOE (Town *et al.*, 1998; Song *et al.*, 1998; Haines *et al.*, 1996; Brindle *et al.*, 1998; Crawford *et al.*, 1998; Farrer *et al.*, 1998; Scott *et al.*, 1996).

1.2 Similar Dementias

Alzheimer's disease is similar to several other dementias in its cognitive impact, as well as its pathological effect. The most common Down's Syndrome, or Trisomy 21, a triplication of the chromosome 21, has a very strong pathological similarity to AD. This is attributed to the excessive production of APP, causing a downstream increase in Aβ production (McKenzie *et al.*, 1996; Lennox *et al.*, 1988) . In addition, there is also an increase in the expression of another protein, S100β, encoded on chromosome 21 (Griffin *et al.*, 1998; Castets *et al.*, 1997; Van Eldik and Griffin, 1994; Jorgensen *et al.*, 1990).

Dementia pugilistica is a condition used to characterize the pathogical dementia associated with repetitive blunt force trauma often seen in boxers. In dementia pugilistica, there is evidence of neurofibrillary degeneration and occasionally senile plaques (Buee *et al.*, 1994; Clinton *et al.*, 1991; Dale *et al.*, 1991; Roberts *et al.*, 1990; Rudelli *et al.*, 1982). In both these dementias, the onset of the pathology is rapid and severe.

1.3 Cytokine Cycles and Astrocytes

Immune responses have been observed in Alzheimer's disease as a consequence of cellular insults and death. Among the first observations was the presence of immunoglobulin G presence in senile plaques (Ishii *et al.*, 1975). In addition, McRae and Dalhstrom (1991, 1992) found that CSF contained antibodies to endogenous proteins found in the brain. While this data suggests that there is a dysfunction of the blood-brain barrier, a more plausible explanation is the increase in immune response, as indicated by increased inflammatory activity (McGeer and McGeer, 1995). In plasma, in response to injury, there is usually an acute increase in selected proteins such as α1 antichymotrypsin, α-2 macroglobulin, amyloid P, sulphated proteoglycans and complement factors (Abraham *et al.*, 1988; Bauer *et al.*, 1991; Castano *et al.*, 1994; Kisilevsky, 1991; Abbas *et al.*, 1991; Emmerling *et al.*, 2000).

Reactive astrocytes and microglia produce cytokines which are the most prevalent indication of inflammatory activity in AD (Mrak *et al.*, 1995; Griffin *et al.*, 1989; Woods *et al.*, 1993; Cacabelos, 1994; Dickson *et al.*, 1993). Cytokines are a large group of immune-related proteins which can also be produced by macrophages (Perry, 1994). Among cytokines that are elevated in Alzheimer's disease are interleukins -1 (IL-1β), -3 (IL-3), -6 (IL-6) and TNF-α. While the specific activity is not completely defined, they are elevated in

response to cellular insults. For example, IL-1β is upregulated by the presence of Aβ. In addition, microglia are known to remove plaque deposits and are commonly associated or localized to the vicinity of senile plaques (Griffin *et al.*, 1995; Stalder *et al.*, 1999). IL-1β also appears to promote APP production, in addition to other proteins, like S100β (Sheng *et al.*, 1996b). Microglia upon activation can remove Aβ deposits, though there is a dispute regarding its effectiveness, or possibly its detrimental activity (Stoll and Jander, 1999). Most researchers generally consider cytokine activation as being deleterious in the brain, especially in Alzheimer's disease (Mrak *et al.*, 1995; McGeer and McGeer, 1995).

1.4 Metal Binding Proteins with Neuronal Regulatory and Function

1.4.1 S100β Biology

S100β is a member of the large S100 family, which is loosely related to the calcium binding calmodulin family. S100β is expressed predominantly in the central nervous system, though it is also used as a tumour marker in cancer. S100β, which is functional as a dimer, can bind four calcium ions with an affinity of 3×10^3 M^{-1}. Zinc binding sites are distinct from the calcium domains on S100β. There are 6-8 known positions based on a Cys-x-Cys and Cys-X-X-Cys motif with a K_a of approximately 10^{-6}. Zinc binding appears to also reduce calcium binding activity (Hasler *et al.*, 1998).

S100β is produced by astrocytes, can be induced by IL-1β and it is secreted into the extracellular space. In addition, it is known to bind a limited number of proteins including fructose-1,6-biphosphate, aldolase, calponin, tau, tubulin, caldesmon, neurocalcin, GAP43, GFAP, CapZ (Kilby *et al.*, 1997), p53 (Baudier *et al.*, 1992; Rustandi *et al.*, 1998), nuclear Ser/Thr protein kinase (Millward *et al.*, 1998), bHLH transcription factor (Baudier *et al.*,

1995). S100β has numerous functions including promoting neurite extension, proliferation of melanoma cells, inhibition of PKC-mediated phosphorylation, axonal proliferation and astrocytosis, guanylate cyclase activation (Pozdnyakov *et al.*, 1997). Of interest is that S100β levels in the brain increase with age, potentially predisposing the brain to low-level pathogenic characteristics associated with neuritic outgrowth, and metal toxicity.

1.4.2 S100β Relationship to AD

The gene for S100β is located on chromosome 21, distal to the APP gene. In Down's syndrome and Alzheimer's disease, its expression is upregulated by up to 24 fold. As it is known to promote neurite extensions, it may promote the neuritic dystrophy observed in the disease process. Furthermore, as a moderate metal binding protein, it may provide a labile source of zinc and calcium, which are metals believed to be involved in AD pathogenesis.

1.4.3 Metallothionein III

Metallothionein III (MT-III) when originally discovered was called growth inhibitory factor due to its characteristic effect on inhibiting neuronal sprouting (Uchida *et al.*, 1991). It is a member of the copper (maximum four ions) and zinc (maximum three ions) binding metallothionein (MT) family (Uchida *et al.*, 1991; Vasak *et al.*, 2000; Bogumil *et al.*, 1998; Palmiter *et al.*, 1992). MT-III has a sequence similarity of 70% to other MTs. Whereas MT-I and MT-II are 61 amino acids in length, MT-III has an additional threonine at position 5 and a glutamate rich sequence at amino acids 54-58 (Uchida *et al.*, 1991). It does maintain the 20 cysteines found in the MT family which are the sites for metal binding. Metallothioneins play an important role in maintaining copper and zinc homeostasis, as well

as removal of heavy metals such as cadmium and mercury (Templeton and Cherian, 1991; Maret and Vallee, 1998).

Unique to the metallothionein family is the neurotrophic capacity of MT-III (Uchida *et al.*, 1991; Erickson *et al.*, 1994; Bruinink *et al.*, 1998), as well as the near exclusive expression of the protein in the brain (Tsuji *et al.*, 1992; Kobayashi *et al.*, 1993; Moffatt and Seguin, 1998). The neuroinhibitory function of MT-III has been localized to the first 26-32 amino acids (Uchida and Ihara, 1995; Sewell *et al.*, 1995) which can be ablated with the deletion or substitution of the Cys-Pro-Cys-Pro sequence (at residues 6-9) (Sewell *et al.*, 1995). MT-III expression was originally believed to be in astrocytes, but more recently, based on mRNA expression, it was demonstrated that neurons express MT-III (Yamada *et al.*, 1996; Arai *et al.*, 1997; Masters *et al.*, 1994; Yuguchi *et al.*, 1995; Velasquez, 1999). MT-III mRNA is induced by injury. Stab wound studies in rat brains demonstrated increased MT-III expression up to 28 days post-trauma was observed (Hozumi *et al.*, 1995). MT-III regulation is not currently known, though conventional MT regulators such as dexamethasone or heavy metals fail to induce MT-III expression. Potential downregulators of MT-III are the astrocytic cytokines IL-1β and IL-6 (Hernandez *et al.*, 1997;Uchida, 1998).

1.4.4 MT-III Relationship to AD

In AD, the increase in neurotrophic activity may be attributed to a reduction in MT-III (Uchida *et al.*, 1988, 1991). There is a lack of consensus on MT levels based on limited analysis of protein and mRNA studies (Uchida, 1991; Erickson *et al.*, 1994; Sewell *et al.*, 1995). Furthermore, as metallothioneins represent a major binding protein for zinc and copper, metal homeostasis may also be disrupted if there is a downregulation of the protein.

MT-III is most abundant in the hippocampus and dentate gyrus where downregulation of MT-III may present as zinc homeostatic disruption and uninhibited neuronal sprouting (Hesse, 1979; Golub *et al.*, 1983; Frederickson *et al.*, 1990; Constantinidis, 1990; Wenstrup *et al.*, 1990; Samudralwar *et al.*, 1995; Tully *et al.*, 1995). MT-III downregulation in mice does not result in AD-like pathology, but are more prone to neuronal deficits and injury than control mice (Erickson *et al.*, 1997). CA3 neurons lacking MT-III were more susceptible to kainate induced epileptic seizures than controls. This may be a result of disruption of zinc regulation. MT-III is required to regulate vesicular zinc during sustained neuronal firing by facilitating the recycling of zinc or serving as a reservoir. In the absence of MT-III, vesicular zinc in the mossy fibers or in other zinc-containing terminals may decline rapidly during prolonged stimulation and consequently result in reduced capacity to prevent seizure activity (Erickson *et al.*, 1997). MT-III could also play a role in modulating and protecting against glutamate- and NO-associated signal transduction toxicity (Montoliu *et al.*, 2000).

1.5 Objective of Research

The objective of this thesis is to examine the roles S100β and metallothionein-III play in regulating neuronal activity and survival. The research will focus on the identification of their expression in Alzheimer's disease and how they interact with proteins central to the development of the disease. Specifically, the involvement of the metal binding proteins with tau and Aβ will be evaluated. These studies will also address the possibility that zinc, a target for both proteins is involved in the pathogenic process. In addition, it will be constructive to examine the regulation of the proteins themselves, particularly, astrocytic cues, such as cytokine levels in aging and AD, and how this may affect S100β and MT-III

levels. The primary goal of this research is to determine what contribution MT-III and S100β, metal-binding proteins with neurotrophic activity, have with respect to pathological events observed in Alzheimer's disease and how this may modulate the progression of Alzheimer's disease.

1.6 Hypothesis

S100B and MT-III experimentally modulate neuronal function and survival.

1) MT-III levels are decreased in AD brains;

2) S100ß will bind to tau and increase neuritic extension;

3) MT-III is likely to sequester zinc, while S100ß increases the labile form. The net effect of decreased MT-III and increased S100ß is in vitro promotion of zinc-induced Aß aggregation which mimics a pathological event observed in AD;

4) Astroglial cells, which produce MT-III and S100ß, also produce more IL-1ß in aging. IL-1ß, in turn, regulates MT-III and S100ß expression.

Chapter 2

Authors - Craig A. Mizzen, Nicholas J. Cartel, Wai Haung Yu, Paul E. Fraser and Donald R. McLachlan.

Title - Sensitive detection of metallothioneins -1, -2 and -3 in tissue homogenates by immunoblotting: a method for enhanced membrane transfer and retention.

Journal – Journal of Biochemical and Biophysical Methods (1996) 32:77-83.

Contribution to Paper –

1) Produced polyclonal MT-III antibody used in this study.
2) Performed half of immunoblots.
3) Helped write and edit manuscript.

Significance of Article to Thesis –

This paper provided crucial methods for the analysis of MT-III, as well as established the effectiveness of the antibody. This antibody was used for MT-III expression in Alzheimer's disease, as well as being used by other groups in their studies and publications.

2.1 ABSTRACT

Standard immunoblotting procedures were unable to detect metallothioneins-I and 2 (MT-1, MT-2) and metallothionein-3 (MT-3)/growth inhibitory factor (GIF) in unfractionated brain homogenates. We have developed a novel process which involves the inclusion of 2mM $CaCl_2$ in electrophoretic transfer buffers and glutaraldehyde fixation following transfer to either nitrocellulose or polyvinylidene difluoride (PVDF) membranes. Using commercial MT antibodies and a specific MT-3 polyclonal antibody raised in our laboratory, we have been able to detect all three MTs on both membrane types with a detection limit of approx. 10 ng, for MT-1 and MT-2. Nitrocellulose membrane pore size had no noticeable effect on detection sensitivity. These modifications enable more sensitive MT detection than previously described blotting methods. In addition, this technique eliminates the need for indirect monitoring approaches and simplifies quantification since sample fractionation or enrichment are not required.

2.2 INTRODUCTION

Metallothioneins (MT) are a family of ubiquitous, small, metal-binding proteins with apparent roles in cellular responses to metal exposure and other stimuli (Kojima, 1991; Foulkes, 1991; Templeton and Cherian, 1991;Bremner, 1991). A recently described metallothionein isoform, growth inhibitory factor (GIF), also designated MT-3, has been implicated in the pathogenesis of Alzheimer's disease (AD) in which the levels of GIF are reported to be decreased in brain tissue (Uchida *et al.*, 1991). However, the downregulation of MT-3 (GIF) in Alzheimer's disease were not observed in all cases (Erickson *et al.*, 1994) when protein levels were measured by indirect techniques. This raises questions concerning, the reliability of direct detection of MT by immunological techniques.

To investigate this possibility further, we attempted to develop a direct immunoblot assay for GIF. During the course of our work, we discovered that GIF bound weakly to nitrocellulose and polyvinylidene difluoride (PVDF) blotting media during electrophoretic transfer and immunodetection such that the majority of GIF was not retained by membranes. These problems also affected the analysis of MT-1 and MT-2 and presumably are responsible for the poor detection sensitivities achieved in previous reports of MT analysis by blotting, (Aoki *et al.*, 1986; Aoki *et al.*, 1991).

Here we describe simple modifications to blotting procedures that permit reproducible and sensitive detection of MT-1, MT-2 and GIF which may also be of use when other low molecular weight, hydrophilic polypeptides are to be analyzed by immunoblotting.

2.3 MATERIALS AND METHODS

2.3.1 Generation of antibodies specific for GIF

Affinity-purified rabbit antisera to a synthetic peptide (GGEAAEAEAF-KC) homologous to the unique carboxyl-terminal insert of GIF was prepared as described by Uchida *et al.* (1991). Briefly, reverse-phase HPLC purified peptide was linked to keyhole limpet haemocyanin and used to immunize several New Zealand white rabbits. Pooled serum was fractionated by ammonium sulfate precipitation and the antibody affinity purified using peptide immobilized on a Sulfo-link (Pharmacia) column.

2.3.2 Preparation of samples

Human brain samples (diagnosed at autopsy as unaffected controls; no observation of Alzheimer plaques or tangles) were obtained from the Canadian Brain and Tissue Bank and stored at -70'C until used. Homogenates were prepared by disruption of tissue in 4 ml of ice-cold 10 mM Tris-HCI (pH 7), 5 mM EDTA, 1 mM PMSF by two 30 sec treatments with the PTA 7 KI probe of a Polytron (Brinkman) at full speed with cooling, on ice in between. Homogenates were clarified by centrifugation at 20 000 *x g* for 45 min at 4°C. Total protein concentration of clarified homogenates was determined using the Bradford assay (Bio-Rad) employing BSA (Calbiochem) as a standard. Equine renal metallothionein (a mixture of MT-1 and MT-2 isoforms) was obtained from Sigma and used without further purification.

2.3.3 Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) using 18% gels in a Bio-Rad mini-gel apparatus. Samples (20 μg total protein for brain homogenates, 0.5 μg equine kidney MT, 1.5 μg peptide-BSA conjugate) in 20 μl of sample buffer (10 mM Tris-HCI, pH 7.5, 10 mM EDTA, 20% (v/v) glycerol, 1.0% (w/v) SDS, 0.005% (w/v) bromophenol blue, 100 mM dithiothreitol) were heated in boiling water for 5 min before loading. Electrophoresis was performed at 200 V at room temperature until the bromophenol blue tracking dye reached the bottom of the gel. Gels to be stained were then immersed in Coomassie brilliant blue R-250 (CBR) staining solution (0.22% CBR, 50% methanol, 7% acetic acid) for 3 or more hours prior to destaining in 50% methanol, 10% acetic acid.

2.3.4 Electrophoretic transfer and glutaraldehyde treatment

Gels to be transferred were equilibrated with transfer buffer for 20 minutes prior to assembly in transfer cassettes. All transfers were performed in a Bio-Rad mini-gel blotting apparatus at 40 V for 45 min to 1 h using, transfer buffer that had been cooled to 4-C with further cooling, provided by the ice-block component of this apparatus. Two transfer buffers were tested: (a) 10 mM 3-cyclohexylamino-l-propanesulfonic acid (CAPS) pH 10.8 in 10% methanol (LeGendre and Matsudaira, 1989) and (b) 25 mM Tris-HCl (pH 8.3), 192 mM glycine in 20% methanol (Towbin *et al.*, 1979) with the addition of 2 mM CaCl2 to each buffer as noted in figure legends. Three types of transfer media were tested: Immobilon-P (Millipore) PVDF membrane and nitrocellulose membranes with 0.05 and 0.45 μm diameter pores (Schleicher and Schuell). Each transfer experiment incorporated one of the above as a

primary membrane for immunodetection followed by a sheet of 0.05 μm pore nitrocellulose as a secondary or backup membrane on the anode side of the gel. Following transfer, primary membranes were treated for immunodetection as described below. Secondary or back-up membranes were stained for total protein with copper phthalocyanine 3,4',4",4"'-tetrasulfonic acid tetrasodium salt (CPTS, Aldrich) as described previously (Bickar and Reid 1992) to monitor migration of proteins through the primary membrane. Transferred gels were stained with CBR or silver (Morrissey, 1981) to monitor protein transfer efficiency.

To study the efficacy of glutaraldehyde treatment, a series of duplicate blots prepared by transfer in CAPS buffer containing 2 mM CaCl, were incubated with either 0, 0.25, 2.5 or 5.0% (final) glutaraldehyde prior to immunodetection. In our optimal protocol, gels were incubated for 20 min in CAPS buffer containing 2 mM CaCl2 prior to transfer in that buffer at 40 V for 3 h. Following transfer, primary membranes were incubated in 2.5% glutaraldehyde in water for 1h and then washed for 5 min 3 times in phosphate buffer (8.1 mM Na_2HPO_4, 1.2 mM KH_2PO_4, 2.7 mM KCl, pH 7.4) 50 mM monoethanolamine was added to the third wash solution to quench residual glutaraldehyde reactivity.

2.3.5 Immunodetection

All primary membranes were blocked in 317c BSA in Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 154 mM NaCl) for 2 h at room temperature. Membranes were then incubated overnight in primary antibody diluted in 3% BSA in TBS at room temperature. Affinity purified polyclonal antibody to GIF was used at 0.75 μg/ml while monoclonal antibody to polymerized equine renal MT- I and MT-2 (Dako) was diluted 1 in 1000. Membranes were washed in TBS containing 0.05% Tween-20 (Sigma). Affinity purified

secondary antibody horseradish peroxidase conjugates (goat anti-rabbit IgG (Sigma) or goat anti-mouse IgG (Bio-Rad)) was applied at 1 in 1000 dilution in 3% BSA in TBS for 4 h at room temperature. Following, washing as described above, blots were developed in 3.4 mM 4-chloro-l-naphthol, 22% methanol, 0.018% H_2O_2, 15.6 mm Tris-HCI (pH 7.4), 120.2 mM NaCl. Development was terminated by immersion in distilled water.

2.4 RESULTS AND DISCUSSION

Our initial attempts to detect the Growth Inhibitory Factor (GIF) or metallothionein-III in brain homogenates by immunoblotting, were unsuccessful even though the antibody gave robust staining in immunocytochemistry (data not shown). As shown in Fig. 1A and B, human MT- 1, MT-2 and GIF were not detected when brain samples were immunoblotted on PVDF by conventional techniques (lanes 1 and 2). This was not due to a lack of antibody response as demonstrated by the fact that 0.5 μg samples of GIF-antigen peptide conjugated to BSA (lane 3) or equine renal MT (lane 4) were readily detected by the appropriate antisera. Inspection of gels stained with CBR or silver following transfer in either of the unmodified buffers revealed that all detectable polypeptides of less than 30 kDa had been quantitatively transferred (data not shown). However, staining of secondary or backup membranes for total protein from these transfers often revealed that proteins of Mr less than 20000 had migrated through the primary membranes. Thus we reasoned that the failure to detect GIF or other MTs in brain samples was likely to result from poor binding or subsequent elution of the small quantities of these polypeptides present in brain samples from membranes during transfer and immunodetection.

Since it has been shown separately that inclusion of $CaCl_2$ in transfer buffers (McKeon and Lyman, 1991) and glutaraldehyde treatment of membranes (Van Eldik and Wolchok, 1984) enhance calmodulin immunodetection, we examined whether these reagents benefit MT immunodetection. As shown in Fig. 1C and D, the combined use of $CaCl_2$ and glutaraldehyde enabled detection of the MT-1, MT-2 and GIF present in 20 μg, of brain

homogenate protein on PVDF membranes. When used alone these reagents were not as successful as when used in combination. Comparable results were obtained when 0.05 and 0.45 μ pore nitrocellulose filters were used in place of PVDF. A greater fraction of higher molecular weight proteins resisted transfer when Tris-glycine transfer buffer was supplemented with $CaCl_2$ compared to CAPS buffer although MT immunodetection was equivalent. The comparatively weaker signal observed with the GIF antibody (Fig. 1D) is most likely accounted for by the fact that the commercial MT antibody recognizes all metallothionein species and therefore gives a more intense signal (Fig. 1C). The blotting, procedure as described by Aoki *et al.* (1991) did not employ $CaCl_2$ or glutaraldehyde and reported a sensitivity of 400 ng for rat MT-2 detected by ^{109}Cd binding and autoradiography. A similar blotting procedure with immunodetection employing a protein A/colloidal gold conjugate was reported to have a detection limit of 1 μg which could be lowered to 60 ng after silver staining enhancement (Aoki *et al.*, 1991). Fig. 2 shows that the procedure described here detects as little as 10 ng of equine renal MT on either nitrocellulose or PVDF with much less effort. While the data presented here is limited to immunodetection of MT proteins, we suggest that the use of $CaCl_2$ or glutaraldehyde, alone or in combination, may be widely applicable to the electrophoretic transfer and immunoblotting of low molecular weight proteins and in particular those species with a largely hydrophilic sequence.

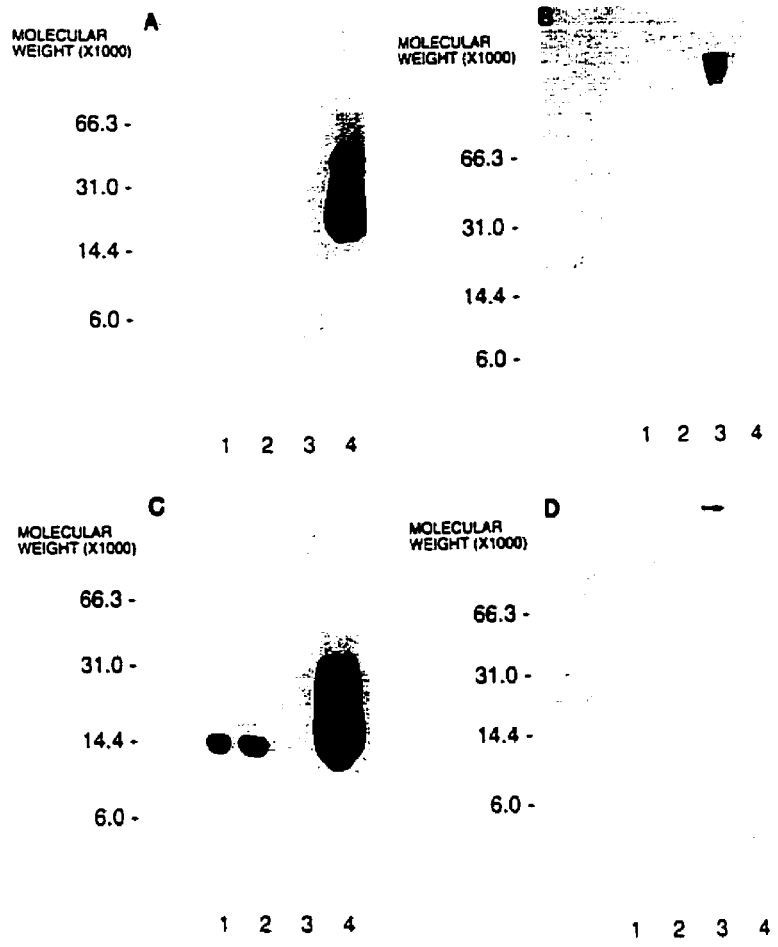


Figure 2.1: Electrophoretic transfer in the presence of calcium chloride followed by glutaraldehyde treatment enhances immunodetection of MT-1, MT-2 and GIF. Brain homogenate samples (20 μg total protein, lanes 1 and 2). GIF peptide-BSA conjugate (0.5 μg, lane 3) and equine renal MT-1 and MT-2 (0.5 μg, lane 4) were electrophoresed in SDS-PAGE gels and immunodetected on PVDF following transfer in CAPS buffer alone (A and B) or following transfer in CAPS buffer containing 2 mM $CaCl_2$ and treatment with 2.5% glutaraldehyde (C and D). In A and C, filters were incubated with antisera to equine MT-1 and MT-2; in B and D, filters were incubated with antisera to GIF peptide. The migration of molecular weight marker proteins is indicated at the left border of each filter.

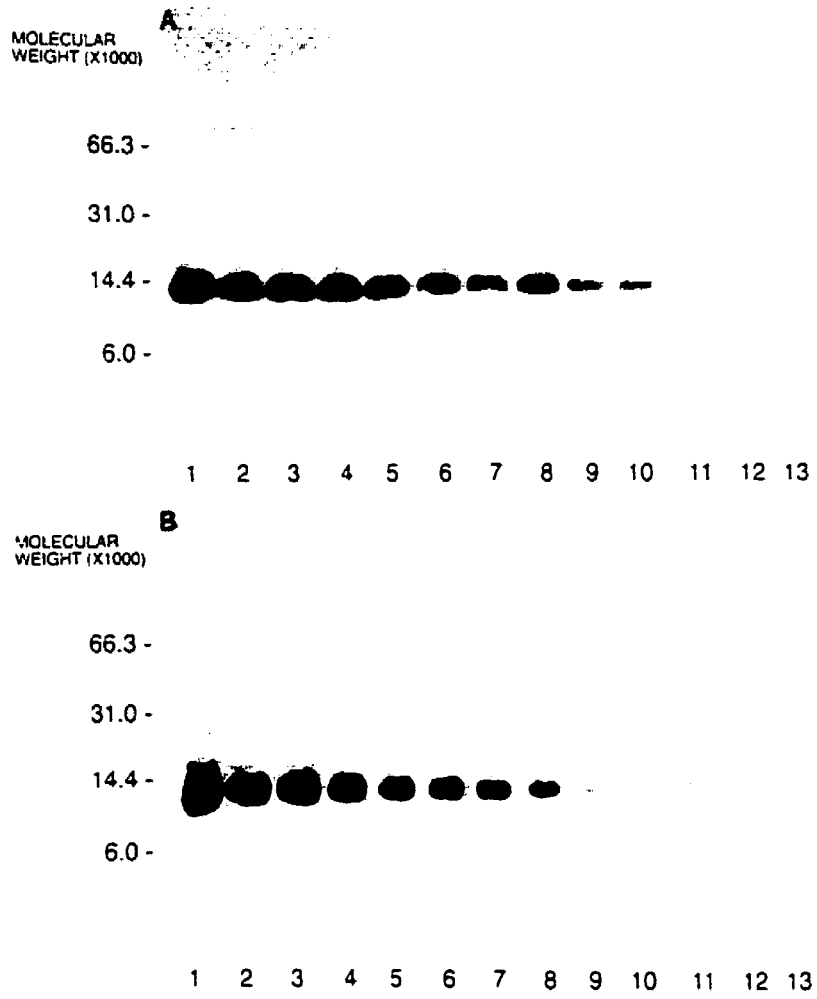


Figure 2.2: Nitrocellulose and PVDF membranes give similar MT detection limits. Aliquots of a serially diluted equine renal MT solution were electrophoresed on gels and transferred with CAPS buffer containing 2 mM $CaCl_2$ to 0.45 μ pore nitrocellulose (A) or PVDF (B) filters which were then treated with 2.5% glutaraldehyde prior to immunodetection with antisera to equine renal MT. The MT loadings were 200, 150, 100, 90, 80, 70, 60, 50, 40, 30, 20, 10 and 1.0 ng in lanes 1 to 13, respectively. The commercial MT in this assay was used as a dry weight rather than the more precise amino acid analysis. Note that this does not represent a standard curve for Fig. 2.1 but indicates a graded sensitivity measurement.

Chapter 3

Authors - W. Haung Yu, Walter J. Lukiw, Catherine Bergeron, Hyman B. Niznik and Paul E. Fraser

Title - Metallothionein III is Reduced in Alzheimer's Disease

Journal – Brain Research 2001 Mar 9; 894(1):37-45

Contribution to Paper –

4) Performed all protein expression analysis and RNA/DNA analysis in collaboration with Dr. Lukiw.
5) Wrote and edited manuscript.

Significance of Article to Thesis –

This article provided extensive evidence for a defect in the expression of MT-III in Alzheimer's disease. While other scientists have examined MT-III levels in AD, this paper is the only one to examine all levels of expression from DNA mononucleosome chromatin to protein. Furthermore, under greater stringency (sample size and post-mortem times), this paper goes beyond the work of others in the analysis of MT-III in AD.

Notes for Chapter:

See Appendix I for additional data and revisions.

Following publication of the paper, a mistake was noted in identifying cells in some of the sections and has been rectified in the erratum. It was noted that MT-III expression in control tissue is present in glial cells. Section 3.4.1, page 59 should read "Immunoreactivity for MT-III was relatively abundant in the control tissues and localized primarily in glial cells (Fig. 3.4A,B)." Furthermore, Fig. 3.5 should have on the X-axis, first label, "# cells" and not "# neurons".

Values represented in this chapter are based on mean +/- 1 standard deviation.

The publisher has been contacted regarding the correction.

3.1 ABSTRACT

Metallothionein III (MT-III) is a functionally distinct member of the metallothionein family that displays neuroinhibitory activity and is involved in the repair of neuronal damage. Altered expression levels of MT-III have been observed in Alzheimer's disease (AD) which has led to suggestions that it could be a mitigating factor in AD-related neuronal dysfunction. However, conflicting results have been reported on this issue which may be due to methodological differences and/or sampling size. In the current study, we have assessed MT-III expression in a large number of AD cases through the quantification of mRNA as well as by immunohistochemistry and western blotting using a MT-III specific antibody. The results of this comprehensive study indicate that the mononucleosome DNA encoding MT-III is occluded preventing transcription and that message levels are reduced by ~30%. In addition, protein levels were specifically decreased by ~55% in temporal cortex. These data support the conclusion that MT-III is significantly downregulated in AD and may contribute to the loss of its protective effects and/or repair functions that lead to an exacerbation of the pathogenic processes.

3.2 INTRODUCTION

Alzheimer's disease (AD) is characterized by neuronal loss that is accompanied by neuritic plaques and neurofibrillary tangles (Berg and Morris, 1994). Many of the remaining neurons display an aberrant neuritic sprouting which may reflect a compensatory mechanism following reduction in the neuronal population (Griffin *et al.*, 1989). The underlying relationship between the various pathological factors has yet to be fully resolved but several potential contributors to the disease process have been identified. For example, presenilins mediate the processing of the amyloid precursor protein to yield the β-amyloid protein (Aβ) which is the principal component of neuritic plaques (Citron *et al.*, 1997; De Strooper *et al.*, 1998).

In addition to the obvious pathological proteins, other elements have been examined with regard to their association with AD. One potential factor is metallothionein III (MT-III) which is a member of the zinc- and copper-binding metallothionein family. The properties of MT-III were originally characterized by Uchida and Tomonaga (1989) with the functional sequence identified and termed growth inhibitory factor (GIF) (Uchida *et al.*, 1991). They determined that MT-III is expressed exclusively in the central nervous system. It has two inserts at position 5 and 55-60 and possesses neuroinhibitory properties that make it unique from other metallothioneins.

MT-III is a brain-specific protein that was originally identified as an astrocytic component but subsequent studies have demonstrated expression in zinc-sequestering neurons (Masters *et al.*, 1994). As with other members of this family, MT-III binds copper and zinc ions with a capacity of 3-5 zinc and 1-4 copper atoms bound within the Cys-X-Cys

and Cys-X-X-Cys zinc finger motifs (Pountney *et al.*, 1994). The ability to bind metals, especially copper, is thought to be the means by which it can reduce free radical activity and facilitate neuronal protection (Schwartz *et al.*, 1995).

Metallothionein III possesses several other unique properties. For example, it is not induced by typical promoters of other metallothioneins such as increased zinc, kainic acid, dexamethasone or lipopolysaccharides (Zheng *et al.*, 1995). In contrast, it is reduced by as much as 30% in the presence of these compounds. Unlike the other metallothioneins, MT-III has also been shown to decrease neuritic sprouting in support of its proposed growth inhibitory function (Uchida and Ihara, 1995). The N-terminal sequence constitutes the functional domain and it is sufficient to produce the growth inhibitory effect that is independent of metal binding. Other studies have demonstrated that zinc deficient cells are more susceptible to MT-III neuroinhibitory activity (Palmiter, 1995), suggesting a metal-independent and a more specialized role for metallothionein III in neurons as compared to other metallothioneins.

In AD, the absence of metallothionein III may be responsible for the initiation of aberrant neuritic sprouting. The absence or a significant decrease of MT-III may compromise neurons and promote neurite extension in an attempt to re-establish synaptic connections. In this instance, a potential cascade may result whereby the affected neurons overextend themselves and eventually die. This study aims to resolve the conflicting evidence of MT-III changes associated with neurodegeneration and provide the framework for the theory of overactive neuritic sprouting and its pathogenic effects in Alzheimer's disease.

However, defining the precise role of MT-III in Alzheimer's disease has been problematic as shown by several conflicting observations. The original observation was that MT-III is downregulated in AD brain tissue samples (Uchida *et al.*, 1991). However, Erickson *et al.*, (1994) contend that the neuronal changes in AD are unrelated to a decrease in MT-III even though there is evidence that MT-III is neuroinhibitory. The current study examines MT-III at both the protein and message level to provide a comprehensive analysis of MT-III in Alzheimer's disease. Examination of protein levels, by Western blotting and immunohistochemistry using a specific MT-III antibody that corresponds to a biologically novel primary sequence provides data and a larger sample size of brain tissue which provides a detailed assessment of AD-related changes of MT-III. This study also examines mononuclesome DNA, messenger RNA of both AD and control cases that demonstrate the upstream effects of MT-III expression in neurodegeneration.

3.3 METHODS

3.3.1 Preparation of Tissue Samples

Tissue samples were obtained from the temporal and frontal cortices of 25 autopsy confirmed Alzheimer's cases and 10 age-matched, non-dementia control subjects. A 20% (weight/volume) brain tissue homogenate in 5 mM phosphate buffer (pH = 7.4) with 1μg/mL leupeptin and 1μg/mL aprotinin was prepared using a Polytron tissue shredder followed by standard homogenization. Samples were maintained at 4°C between homogenizing cycles and spun at 11,500 x g for thirty minutes to remove cellular debris. Supernatants were used for quantification of metallothionein III and total metallothionein. For western blotting, aliquots of total protein (100μg) were lyophilized and stored at -20°C.

3.3.2 Antibody Production

A peptide corresponding to the unique MT-III sequence - KGGEAAEAEAEK (residues 52-64) - was linked to maleamide activated keyhole limpet haemocyanin via a carboxyl terminal cysteine. This antigen was used to produce a polyclonal antibody as previously described (Mizzen *et al.*, 1996).

3.3.3 Gel Electrophoresis and Western Analysis

Total protein samples (100μg) were combined with 20μL of Laemmli buffer containing 10% dithiothreitol (DTT), heated to 100°C for 5 minutes separated on a 10-20% Tricine gel (Novex). To quantify the relative amount of MT-III, the total protein was determined by analysis of the Coomassie-stained samples (in additional to protein analysis)

which was used to normalize the values obtained by Western blotting with MT-III specific and the generic MT antibody, which recognizes MT I and II isoforms.

3.3.4 Coomassie Blue Reagent (CBR) Staining

Following electrophoresis, the SDS-PAGE gel was stained with CBR solution followed by destain until all background was eliminated. The gel was then mounted and dried between two pieces of cellophane membrane sheets. The various samples were examined for total protein by scanning the dried gel using a high-resolution scanner. The digitized image was consequently analyzed using NIH Image 1.59.

3.3.5 Immunoblot Staining

Following the protocol established by Mizzen *et al.*, (1996), gels were incubated with 2mM $CaCl_2$ and then transferred to 0.45μ PVDF (polyvinylidene fluoride) membranes (Millipore) by standard procedures. Due to the poor binding of metallothioneins to membranes, the proteins were fixed by exposure to glutaraldehyde (2.5% w/v). Residual gluteraldehyde was removed by successive washing followed by a quenching with monoethanolamine. Non-specific binding was eliminated by blocking with 5% skim milk and the blots incubated overnight with the MT-III specific primary antibody or a monoclonal anti-metallothionein antibody to determine the levels of MT-I and MT-II (Dako clone E9, 1:1000). Immunoreactivity was visualized with peroxidase-conjugated secondary antibodies (Boeringher-Mannheim) and developed by enhanced chemiluminescence (Amersham). Films were scanned and quantitated using NIH Image software based on pixel density (n=10 / lane).

3.3.6 Immunohistochemical Analysis of Total Metallothionein and MT-III

Tissue from three control and three autopsy-verified Alzheimer brains from the right medial temporal region were stained with the MT-III specific antibody as well as for total MT-I/II for comparative purposes (DAKO, clone E9, 1:100). Formalin-fixed tissue was deparaffinized, treated with a 3% hydrogen peroxide and non-specific binding blocked by incubation in 3% BSA. Sections were incubated overnight at 4°C with the appropriate antibody in 0.3% Trition X-100 in phosphate buffered saline. Immunoreactivity was visualized with biotinylated secondary antibodies (Boeringher-Mannheim) and peroxidase-conjugated streptavidin (DAKO) with colourimetric development using diaminobenzidine (0.5mg/ml, Sigma) and hydrogen peroxide. Slides were counterstained with haemotoxylin (Sigma) and the ratio of immunopositive versus unstained cells (neurons identified using the haemotoxylin counterstain) were determined by counts from several different fields of each AD and control case.

3.3.7 RNA Message Extraction, Probe Selection Northern Analysis

Neocortices were dissected in the frozen state (-45° C) in the presence of PMSF (20 μM) and human placental ribonuclease inhibitor (1U/mL) to minimize non-specific RNA and protein degradation. Total cellular RNA was isolated by a method modified from Chomski (Chomczynski, 1993) using TRIzol Reagent (Gibco BRL). Probes were generated by cloned genomic or cDNA fragments, or by PCR utilizing primers derived from the GenBank DNA sequences (Lukiw *et al.*, 1996a; Lukiw *et al.*, 1996b). Probes were typically radiolabelled to specific activities of ~10^9 dpm/μg using γ-^{32}PdATP or γ-^{32}PdCTP (~3000 Ci/mmol). For

each probe-driven reaction, the radiolabelled probe concentration was in excess (approximately 6 x 10^7 cpm) with respect to target total RNA were hybridized against each 48 cm^2 Northern membrane panel. All samples were standardized against β-actin. Northern transfers and hybridizations were performed on duplicate or triplicate sets of membranes and quantitative autoradiography was carried out using a BioRad GS250 molecular analyzer (Lukiw *et al.*, 1996a; Lukiw *et al.*, 1996b; Palmiter, 1995).

3.3.8 Analysis of Mononucleosomes

Control and AD neocortical nuclei, micrococcal nuclease digestions of isolated nuclei and Southern blotting were performed essentially as described in Lukiw *et al.*, 1994). The promoter probe for MT-III was a random primed radiolabelled primary PCR DNA product spanning from 249 bp to +93 bp of the human MT-III promoter (Lukiw *et al.*, 1995).

3.3.9 Statistical Analysis

Results from immunoblot, cell counts and mononucleosome analysis are expressed as mean±1SD. Differences between AD and control groups were compared using a Student's T-test, whereby $p<0.05$ was considered significant.

3.4 RESULTS AND DISCUSSION

The brain specific metallothionein-III (MT-III) or growth inhibitory factor (GIF) is a contributing factor to neuronal development and may play a role, for example, in the neuritic responses observed in Alzheimer's disease pathology. However, a number of studies have examined the relative expression levels of MT-III in control and AD tissue but with widely varying conclusions (Erickson *et al.*, 1994; Uchida *et al.*, 1991). The reasons for these contradictory observations is unclear but may be due to a number of factors such as differences in the antibodies employed and/or sampling-dependent variations as previously suggested (Carrasco *et al.*, 1999; Erickson *et al.*, 1994; Uchida *et al.*, 1991). In an effort to resolve this problem, we have examined MT-III levels in as comprehensive manner as possible. Using a large sample size of AD and control tissues, MT-III expression was assessed at the protein level by immunocytochemistry and Western blotting. These findings were supported by additional evaluation of MT-III message and transcriptional regulation.

Protein levels were evaluated using an MT-III specific polyclonal antibody raised to a unique C-terminal sequence which we and others have demonstrated only recognizes MT-III (Mizzen *et al.*, 1996). To increase the statistical significance of our study, a large sample size was employed. Both the frontal and temporal cortex were examined from a total of 25 AD and 10 control tissues. The samples were controlled for age variation as much as possible with the AD cases ranging from 58-91 years for an average of 75.5±10.2. Comparable control samples were collected which varied from 46 to 92 years of age with an average of 70.6±18.0. The individual samples were standardized in terms of total protein content and levels of the combined metallothioneins I and II (MT-I/II) were examined by immunoblotting and compared to the expression of MT-III only. Representative western

blots for a subsection (n=5) of the total number of cases (n=25) indicated modest changes in the levels of the total MT-I/II in the AD frontal and temporal cortices as compared to control tissue (Fig.3.1A). More rigorous quantitative analysis of the entire sample set indicated that, when compared to control levels the expression of combined MT-I/II, were slightly but not significantly elevated in AD frontal cortex tissue (Fig. 3.2A). In the temporal cortex the reverse was observed with a reduction of MT-I/II by 13.5% in the AD cases with all samples standardized to the amount of protein loaded. However, the comparative level of the combined MT-I/II protein in the affected versus control groups was relatively small and, even in our larger sample size, did not reach statistical significance.

This was contrasted by the MT-III levels which appeared to be significantly decreased in the AD cases as shown by immunoblotting with the specific antisera (Fig. 3.1B). While this reduction was observed in a number of cases, we did note that the frontal tissue samples exhibited an empirically greater sample-to-sample variation. This was not observed in the temporal cortical expression of MT-III which displayed consistent decreases in the AD tissues and noticeably less variation between individual cases. Quantitatively, the MT-III levels were decreased an average of 29.4% in the frontal cortex of AD brains when compared to the age matched controls (Fig. 3.2A). As noted, intersample variations in the control and AD frontal cortices resulted in a lack of statistical significance ($p<0.10$). It is therefore difficult to ascertain if these changes are related in any meaningful way to AD pathology. In the temporal cortex more consistent differences between AD and control were observed due to the lack of inter-sample variations. The immunoblotting results for MT-III expression revealed a significant and considerable decrease of 55.5% ($p<0.01$; Fig. 3.2A). These findings were supported by the independent measure of MT-III levels standardized by

the CBR methodology that indicated a reduction of 31% in the frontal and 54.7% in the temporal cortex (data not shown). The AD-related decrease in MT-III levels was also observed by comparison of the ratio of total MT-I/II to MT-III alone. The relative ratio between the two metallothionein groups in the frontal cortices was 25.2% in AD as compared with 37.0% in the control cases (Fig. 3.2B). A similar trend was observed in the temporal cortex with MT-I/II-to-MT-III ratio of 20.5% in AD and 39.9% in unaffected individuals. This quantitative evaluation of metallothionein expression indicates that MT-III is significantly decreased in our large selection of sporadic Alzheimer cases.

3.4.1 Immunohistochemical Analysis of MT-III positive Cells in Human Brain

Western blotting analysis of metallothionein levels provides a relative measure of total protein within a given section of the brain. The observed variation from case-to-case may be due to differences in expression within a small population of cells (*e.g.*, reactive astrocytes). Therefore small numbers of cells overexpressing the various metallothioneins could lead to an underestimation of the more specific decreases (or increases) between individual cells. To support our Western blotting observations and to obtain information on the specific cellular expression of these proteins, we examined control and AD tissue by immunocytochemistry using both generic MT antibodies and an MT-III specific antisera. For these immunohistochemical analyses, the right medial temporal cortices from three control and three AD samples were examined. MT-I and MT-II reactive cells in both AD and control tissue appeared to be comparable both in number and intensity (Fig. 3.3). MT-I/II immunoreactivity was most evident in astroglia as has been previously demonstrated (Carrasco *et al.*, 1999). In the majority of AD cases, an increased MT-III staining was

observed in the reactive astrocytes which may account for the slight differences observed by Western blotting for the affected and control groups.

Immunoreactivity for MT-III was relatively abundant in the control tissues and localized primarily to neurons but also observed in glial cells (Fig. 3.4A,B). This corresponds with Velazquez *et al.* (1999) who found that MT-III expression within zinc bearing neurons. This readily observable staining was not paralleled in the AD cases. In all affected cases that we examined, the MT-III-positive cells were severely decreased both in total number and intensity of staining (Fig. 3.4C,D). Some limited MT-III staining was observed in reactive astrocytes present in the AD samples as well as a single control. This observation may provide a partial explanation for the apparently contradictory findings that have been reported for MT-III levels in AD. That is, individual astrocytic responses vary and therefore the total MT-III as determined by western blotting would show a similar variation, particularly in a small sample size. As previously stated, there has been extensive discussion regarding the expression of MT-III in neurodegenerative diseases such as AD. This debate also extends to the location of expression with Uchida *et al.*, (1991) originally concluding that MT-III was an astrocytic protein. This was countered by Masters *et al.*, (1994) who found that zinc bearing neurons in the hippocampus appeared to be the major source of MT-III. More recently, Sogawa *et al.*, (1999) have demonstrated expression of MT-III mRNA in glial cells in mice. In our study, we clearly observed MT-III expression in astrocytes particularly in the AD cases and this likely represents the principal source of the MT-III observed by western blotting (see Fig. 1). The increased number of reactive astrocytes in AD would also account for the discrepancy between the fact that by immunocytochemistry almost no staining was observed but easily detectable levels were found by western blotting.

The increased MT-III expression in astrocytes would therefore also overestimate the severe reduction that was evident by our histological analyses.

The reduction in AD-related MT-III staining was further supported by quantitative analysis based upon counts of immunopositive cells. A total of 20 fields were surveyed for each of the control and AD samples and were initially assessed in terms of total number of cells present (based on hematoxylin counterstaining). This revealed a relatively uniform density with an average of 104.6±32.8 cells/field in the AD cases and 95.2±16.4 for controls. In terms of total MT-I/II immunoreactivity, a combined average of 12.8±3.2 positive cells were observed in the AD cases (Fig. 3.5). This was comparable to the total number of 15.1±5.7 cells per field that were found in the control cases. This translates to approximately 12.1% MT-I/II positive cells in the AD samples as compared to 15.9% in controls. This modest difference is consistent with the quantification of the immunoblotting data and confirms that there is little or no change in these two proteins. For the MT-III specific staining, analysis of the immunopositive cells in the control samples revealed that approximately 21.1% (25.1 of the total 119.3 cells) showed discernable staining. In contrast, the virtual absence of MT-III staining in the AD cases was reflected in the quantification where only 5.6% of the total number of cells displayed even modest immunoreactivity (Fig. 3.5). Overall this represents a decrease of 91% MT-III immunoreactivity in AD tissue compared to the control cases. The decreased MT-III level is consistent with the western blotting results but the magnitude of the reduction is far greater when measured by immunocytochemistry. The potential reason for this discrepancy is that each cell may not produce the same amount of MT-III. Astrocytes in AD may be producing more MT-III and compensate for decreased MT-II expression in AD.

3.4.2 RNA and DNA Analysis of MT-III

To support our observation of a decreased MT-III expression, quantification of monomer nucleosome and total RNA message was performed and compared in both normal and AD cases. Five samples (superior temporal lobe, Brodmann Area 22) were used and were selected for their low post-mortem times (3.0hr±1.54 for controls; 2.80±1.30 for AD samples) to eliminate, as much as possible, variations due to non-specific degradation (Lukiw *et al.*, 1995). Using β-actin as an internal control, it was found that metallothionein III mRNA in AD tissue are reduced to 39% of controls ($p < 0.01$) (Fig. 3.6A). The range of AD samples was 27.5±6.29 (MT-III mRNA / β-actin mRNA) while the control tissue variation was 70.2±7.56.

Analysis of monomer nucleosomes provides insight into the availability of chromatin for transcription. Micrococcal nuclease (MN) were dissected from isolated control or AD nuclei as only mononucleosomes, dinucleosomes and larger nucleosomal fragments are accessible to MN activity (Lukiw *et al.*, 1994). In the five samples per group, some variation in the levels of monomer promoter nucleosome formation was observed. Based on pixel analysis of mononucleosome presence in AD brain tissue, the relative amount was 17.95±6.04. In control tissue, there was 42.65±22.40. In comparison between the two groups, there is a significant difference ($p<0.05$) in the amount of mononucleosomes present based on a Student's t-test. In our analysis of metallothionein III-associated mononucleosomes, we demonstrated that AD mononucleosomes are only 40.8% of the levels of control chromatin (Fig. 3.6B). This finding would be consistent with an impairment of the 5' end of the MT-III gene or an occlusion of normal MT-III promoter activity in AD. This

abnormality may explain the decrease in the MT-III transcription in AD and provides further evidence of an overall expression and functional loss of MT-III in Alzheimer's disease.

The current study has examined the protein levels and expression of MT-III in Alzheimer's disease using various techniques. Using a previously characterized polyclonal antibody (Mizzen *et al.*, 1996) we were able to conclude that MT-III is significantly reduced in the temporal cortex of the AD cases examined. We were unable to determine if this is also consistent with a more widespread CNS reduction in AD due to inter-sample variations in the frontal cortex. Furthermore, in AD, the frontal cortex is spared from pathological events and this may also determine the lack of difference between control and AD samples. Similar variations have been reported by Erickson *et al.*, (1994). We feel that our results help to resolve the reported differences on MT-III in AD (Carrasco *et al.*, 1999; Erickson *et al.*, 1994) and support the findings of Uchida *et al.* (1991). The changes in MT-III were most obvious in the decreased level of staining in the temporal cortex in the AD cases.

The multifunctional aspects of MT-III, including its neuroinhibitory and metal binding properties, prompted us to investigate its relative abundance and expression level in AD. Our findings have demonstrated that there is a marked reduction in MT-III suggesting it may have some impact on the disease process. Various studies have examined the role metallothioneins play in metal binding and reduction of free radicals. These investigations have shown that, like other members of the metallothionein family, MT-III is capable of binding specific metal ions. It has also been suggested that this is a requirement for its neuroinhibitory effect (Palmiter, 1995). In addition MT-III is unique in that it does not undergo a similar induction process as compared to other MTs. For example, in the event of neuronal injury caused by stab wounds MT-III is not upregulated in the rat brain (Aschner *et*

al., 1997; Hozumi *et al.*, 1995). This has led to the proposal that MT-III may play an important role in neuronal repair as well as represent part of a protective mechanism to prevent damage. Because it appears to be involved in several different pathways, the changes in MT-III levels could elicit a number of different responses that could promote AD-related pathology. In addition to its ability to alter neuritic sprouting, MT-III may affect other aspects of AD such as the Aβ pathway. It has been proposed that amyloid-β (Aβ) toxicity is accentuated by zinc (Huang *et al.*, 2000) and copper (Huang *et al.*, 1999) via enhanced aggregation of the Aβ peptide. Both metals interact with the metallothioneins and, in the event of MT-III downregulation in AD, it is conceivable that a situation occurs where these metals are more labile and/or abundant. This may produce conditions *in vivo* where the behaviour of Aβ is altered to enhance either aggregation or neurotoxicity. Of course other pathways may also be affected which have yet to be explored. This study provides extensive evidence of a decrease in MT-III at both the protein and RNA level. When placed in context with the other work in this field, our findings indicate a number of possibilities by which the loss of MT-III may be a contributing factor in the pathogenesis of Alzheimer's disease.

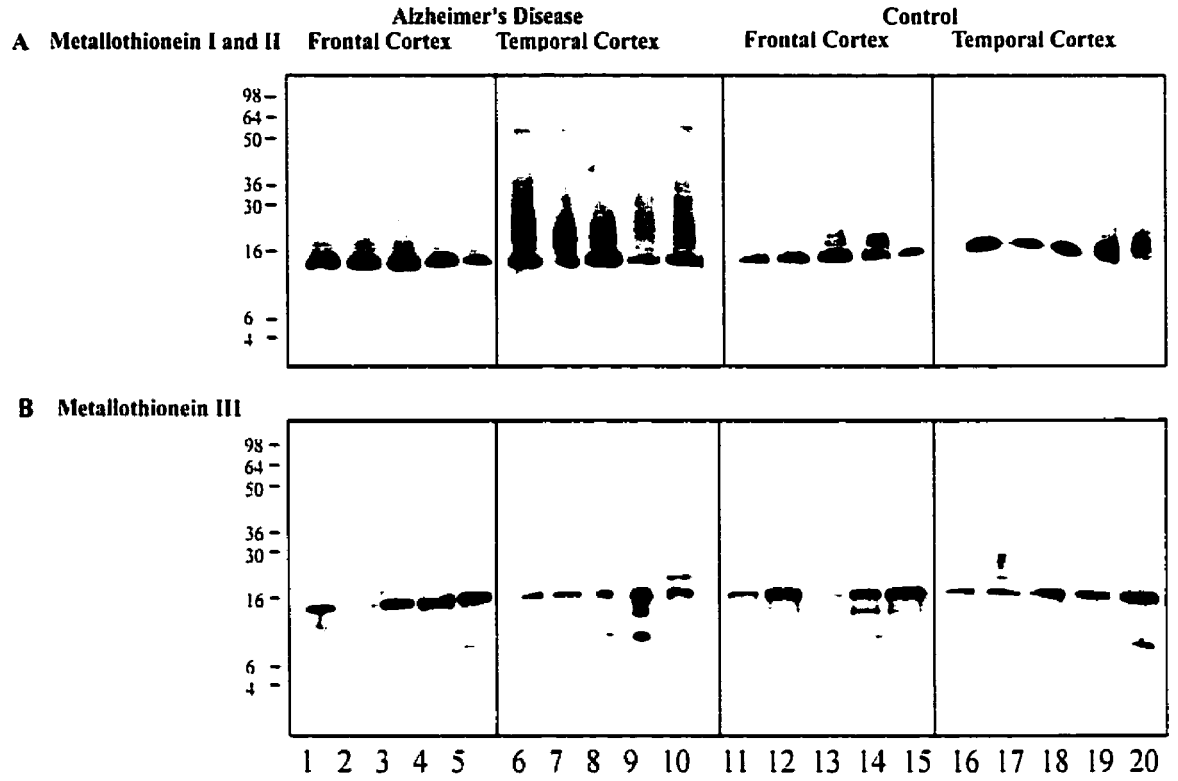


Figure 3.1: Immunoblots of MT-I/II and -III levels in control and AD brains.

Western blots of 5 Alzheimer's disease (lanes 1-10) and age matched control (lanes 11-20) tissue from the frontal (lanes 1-5; 11-15) and temporal (lanes 6-10; 16-20) cortices. In total, 25 AD and 10 control samples were used in this study. Brain homogenates samples probed for metallothionein I/II using a monoclonal antibody (A). Immunoblots for AD and control samples were probed with a MT-III specific antibody (B). The dimer forms of MT I/II (approx 13 kD) and MT-III (approx. 13.8kD) were measured. While the monomer form may also be present it is inconsistent. Intermediary bands may be degradation products and were consequently not measured.

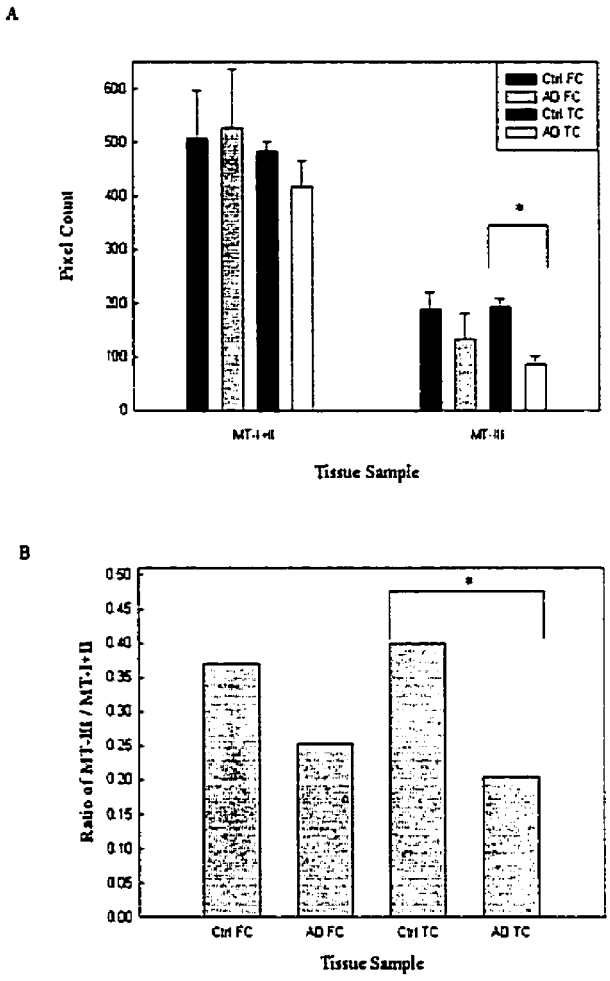


Figure 3.2: Analysis of Western Blots of MT-I/II and -III levels in control and AD brains.

Digital analysis of Western blots of MT-I/II, and MT-III from AD (n=25) and control brains (n=10). Blots were scanned and the digitized images were surveyed 10 times to derive a pixel count per lane. The average per sample is illustrated above along with significantly different samples (when compared to the control tissue using a Student's T-test) noted by an asterisk (*) (A). The ratio of MT-III and MT-I/II, with statistically significant differences between AD and control samples indicated by an * (B).

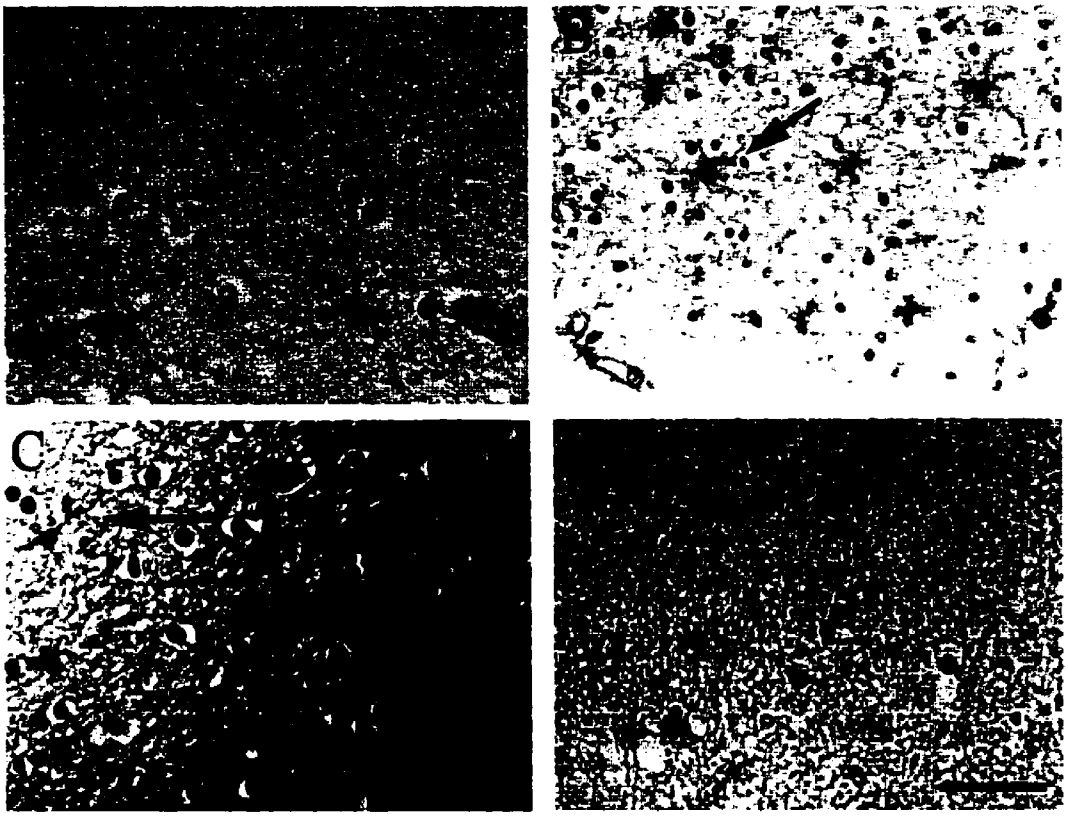


Figure 3.3: Immunoctyochemical Staining of control and AD Brains for MT-I/II.

Immunoreactivity of MT-I/II from control samples (panels A and B) and AD (panels C and D) in brain. Three autopsy-verified AD brains and three age matched controls taken from the temporal cortex were used in this study and two samples from each group are provided here. Sections (10 m) were deparaffinized and stained for MT-I/II. Sections were counterstained with haemotoxylin to permit identification of total neurons as compared to MT-I/II immunopositive cells (arrows). Scale bar = 20 m.

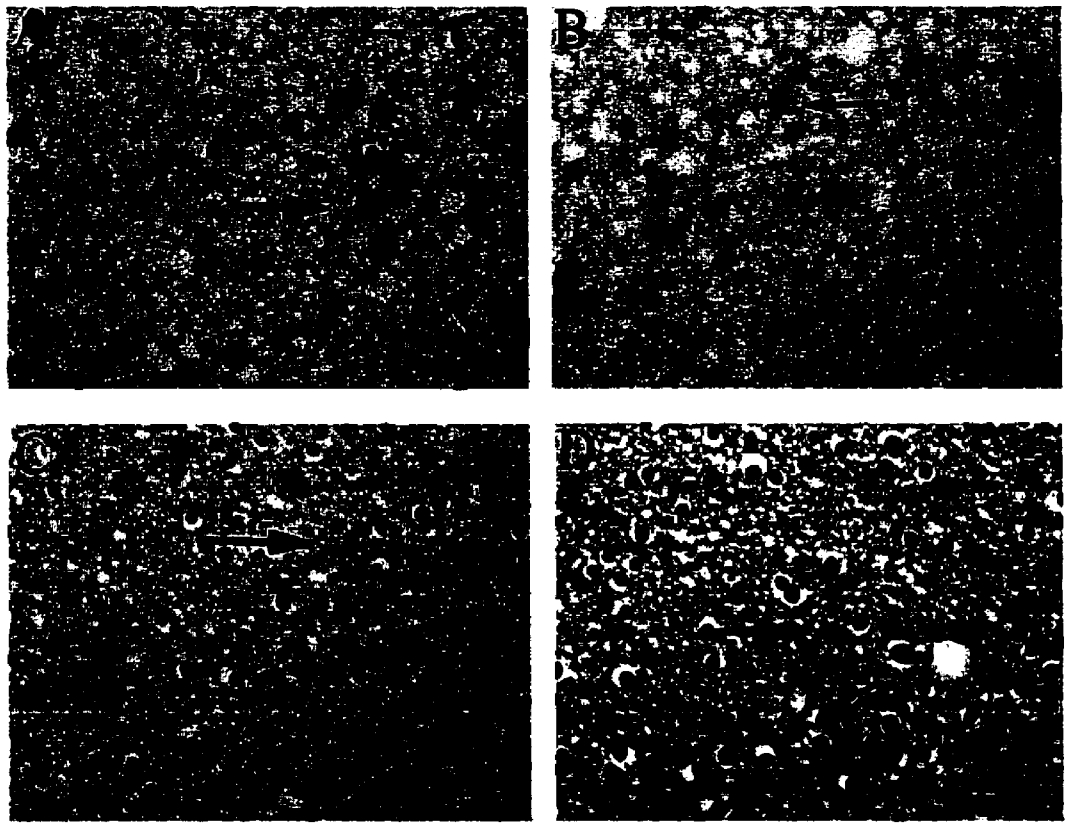


Figure 3.4: Immunocytochemical Staining of control and AD brains for MT-III.

Immunoreactivity of temporal cortical brain samples stained for MT-III in control (panels A and B) and AD (panels C and D) tissue. Arrows are employed to denote immunopositive cells with counterstaining by haemotoxylin in blue. Scale D bar = 20 m.

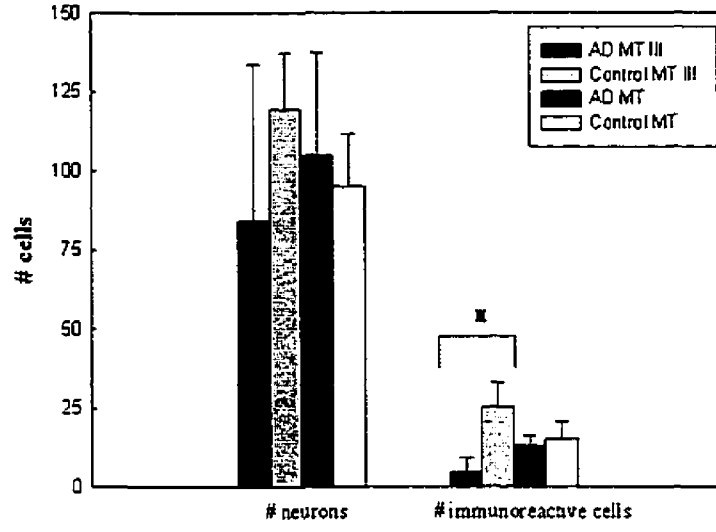
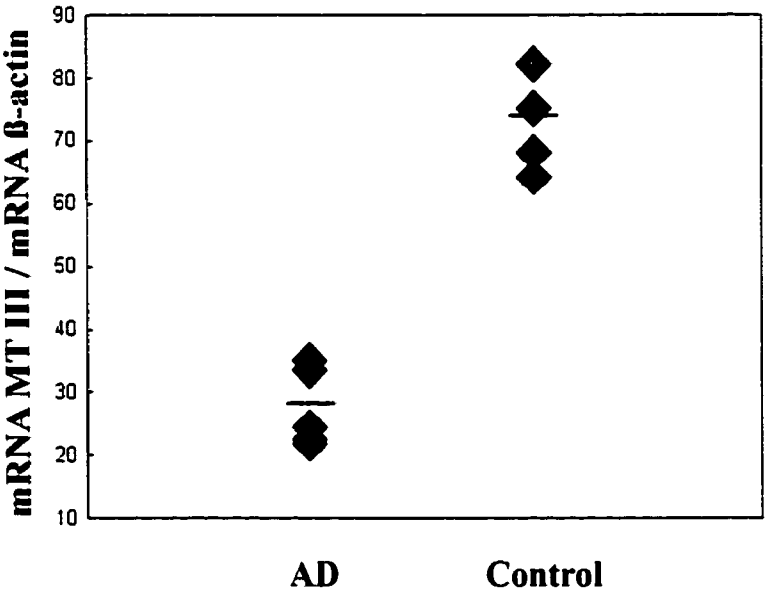


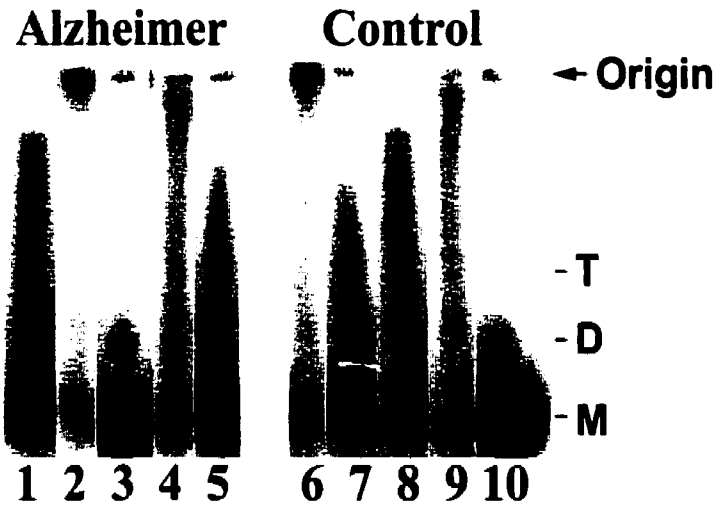
Figure 3.5: Analysis of control and AD brains for immunopositive MT-I/II and –III staining.

Analysis of immunohistochemical staining of MT-III and MT-I/II from brain slices of AD and control temporal brain tissue. For each of the samples, a 250X magnification was used and 20 fields were examined per section to derive a value for both number of immunopositive cells and of total cells per viewing field. Each brain section used was given a value based on total immunopositive and total number of cells present divided by the number of counts (10) made. An average value was derived from the three samples analyzed per group. Based on the Student's T-test, MT-III immunoreactivity is significantly decreased ($p<0.01$) in AD tissue compared to control sample, as indicated (*).

A


mRNA MT III / mRNA ß-actin
90
80
70
60
50
40
30
20
10
AD
Control

B


Alzheimer
Control
Origin
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1 2 3 4 5
6 7 8 9 10

Figure 3.6: MT-III mRNA and nucleosomes in control and AD brain tissue.

Messenger RNA levels of MT-III / mRNA β-actin in AD (samples 1 -5) and control tissue (samples 6 - 10). mRNA of MT-III is significantly decreased ($p<0.05$) in AD tissue when compared to age matched control samples (A). As β-actin may also be decreased in AD due to the loss of neurons, this value may be greater than the 39% decrease as determined by mRNA MT-III / mRNA β-actin. The mean for each group is indicated by a solid line. DNA (promoter nucleosome) data as represented by micrococcal nuclease (MN) isolated from AD (lanes 1 - 5) or control (lanes 6-10) nuclei (B). Only mononucleosomes(M) and not dinucleosomes (D) and larger nucleosomal (T or Origin) fragments are accessible to MN activity (Lukiw *et al.*, 1994). Therefore, this type of chromatin analysis provides insight into the analysis of DNA transcription in AD. Mononucleosomes associated with the MT-III promoter in the 5' region of the MT-III gene are occluded, suggesting that there is an impairment of normal MT-III promoter function in AD.

Chapter 4

Authors - Haung Yu, J. McLaurin, Dun-Sheng Yang and Paul E. Fraser

Title - Modulation of Zinc induced Amyloid-β Peptide Toxicity in PC-12 Cells Using the Metal Binding Proteins, Metallothionein III and S100β

Journal – Journal of Biochemistry (submitted Nov 2000)

Contribution to Paper –

6) Performed all experiments
7) Wrote and edited manuscript.

Significance of Article to Thesis –

This article expands on the actions of zinc induction of amyloid fibril formation, a major hallmark of Alzheimer's disease. Through the examination of the zinc-binding proteins S100β and MT-III, this paper identifies that alterations in this protein can affect the rate of fibrillogenesis. In Alzheimer's disease, a decrease in MT-III coupled with an increase in S100β results in an increase in labile zinc, which in turn results in the aggregation and deposition of amyloid onto neuronal cells.

4.1 ABSTRACT

Amyloid β-peptide (Aβ) is the principal component of senile plaques, which are major neuropathological hallmarks of Alzheimer's disease. Aβ is a normal soluble protein that undergoes a pathogenic transition to an aggregated, fibrous form. Recent work has provided evidence that zinc may be a key factor in the promotion and stabilization of Aβ fibrils. This study investigates the *in vitro* mediation of zinc activated amyloid toxicity using NGF-differentiated PC-12 cells with a focus on the phenomenon of zinc mediated amyloid fibrillogenesis and the role of metal-binding proteins. Our findings suggest that there is a synergistic effect upon the co-treatment of zinc and amyloid. While extracellular zinc alone had a half-maximal lethal concentration (LC_{50}) of 87.5μM and the LC_{50} for Aβ alone was 10μM, the LC_{50} for zinc with 10μM of Aβ together was 20μM zinc which was substantially lower than the effects produced individually. The metal binding proteins, metallothionein-III and S100β, relevant to the central nervous system, were also studied to examine their possible contribution to this pathogenic process. Co-incubation of amyloid with either metallothionein-III or S100β significantly altered the toxicity profile. Addition of MT-III attenuated toxicity of zinc alone or zinc with amyloid treated cells. Similar co-treatment with S100β resulted in an increased susceptibility to zinc and amyloid toxicity. Confocal microscopy confirmed the increase in amyloid aggregation on the cell surface of differentiated PC-12 cells with the addition of zinc or zinc with S100β, and the reduction of the fibril deposition with treatment by MT-III. We propose disruptions in S100β and MT-III levels may result in increased zinc induced amyloid aggregation and affect the pathological progression of Alzheimer's disease.

4.2 INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia, affecting 5% of the elderly population over 65 years of age. In addition to the presence of neurofibrillary degeneration and neuronal loss, a key neuropathological component of AD is the deposition of amyloid fibrils forming senile plaques and in the walls of cerebral and meningeal blood vessels (Kislevsky and Praser, 1997; Selkoe, 1999). Amyloid-β is the primary component of the extracellular amyloid deposits. The toxic 40-43 residue Aβ is produced via endoproteolytic activity of β- and γ-secretase (Kang *et al.*, 1987) of the amyloid precursor protein (APP) (Glenner and Wong, 1984; Masters *et al.*, 1985).

Normally, Aβ is a soluble, cellular metabolite associated with various cell types and is present in both cerebrospinal fluid and plasma. Soluble Aβ is present at comparable levels in sporadic AD as compared to unaffected individuals (Barrow and Zagorski, 1991; Haas *et al.*, 1992; Seubert *et al.*, 1992; Shoji *et al.*, 1992). The assembly of amyloid fibrils is a critical event in its toxicity. Furthermore, modulating elements or proteins that promote or inhibit Aβ polymerization may represent a point for therapeutic intervention. Amyloid aggregation is a nucleation-dependent process (Jarrett and Lansbury, 1993; Lansbury, 1997) affected by several factors including its primary sequence (Fraser *et al.*, 1992), peptide concentration (Burdick *et al.*, 1992) and pH (Burdick *et al.*, 1992; Fraser *et al.*, 1991). In addition, membrane lipids (McLaurin *et al.*, 1998) and hydration forces (Yang *et al.*, 1999) within the cell may play important roles in the aggregation process. Other influences can also modulate amyloid fibil formation such as apolipoprotein E (Sanan *et al.*, 1994; Ma *et al.*,

1994; Evans *et al.*, 1995), proteoglycans (Snow *et al.*, 1994; Castillo *et al.*, 1997; McLaurin *et al.*, 1999), α_1-antichymotrypsin (Ma *et al.*, 1994; Abraham *et al.*, 1988; Fraser *et al.*, 1993; Mucke *et al.*, 2000) and metal ions such as Zn^{2+} and Cu^{2+} (Bush *et al.*, 1994a; Huang *et al.*, 1997). Aβ undergoes a process of fibrillization and ultimately deposits into aggregates termed plaques (Inoue *et al.*, 1999) that are insoluble under physiological conditions. Aβ aggregation into cytotoxic amyloid fibers or protofibrils may be a critical factor for neuronal loss in Alzheimer's disease (Pike *et al.*, 1993;Yankner, 1996; Waslh *et al.*, 1999; Hartley *et al.*, 1999).

The role of zinc as a modulating factor has been demonstrated through several *in vitro* investigations (Cuajungco and Less., 1997; Atwood *et al.*, 1999). Biophysically, Zn^{2+} induces rapid and extensive aggregation of synthetic Aβ (Bush *et al.*, 1994a; Huang *et al.*, 1997; Mantyh *et al.*, 1993; Esler *et al.*, 1996). At low concentrations, Zn^{2+} promotes the aggregation of endogenous Aβ in canine CSF which has an identical sequence to that of human Aβ (Brown *et al.*, 1997). Zinc is also elevated in senile plaques (Lovell *et al.*, 1998) and treatment of postmortem AD brain tissue using divalent chelators, such as EDTA, can resolubilize Aβ (Cherny *et al.*, 1999). Additional evidence of an association between zinc and amyloid plaque formation includes Aβ transgenic mice with Zn^{2+} positive staining in mature plaques but absent in preamyloid, non-fibrillar deposits (Lee *et al.*, 1999). Furthermore, preamyloid deposits fail to develop into mature amyloid-bearing plaques in cerebellum when there is a synaptic vesicle deficiency in Zn^{2+} (Lee *et al.*, 1999). While the current debate continues regarding the relevance of zinc to amyloid deposition, the additional evidence that zinc binding to amyloid is centralized on histidine residues at the N-terminal end of amyloid, specifically residues 6, 13 and 14 (Liu *et al.*, 1999; Yang *et al.*, 2000). Zinc,

however, is not normally found in the free form (Cuajungco and Lees, 1997; Aschner *et al.*, 1997; Christensen and Frederickson, 1998; Colvin, 1998; Colvin *et al.*, 2000). It is primarily localized in synaptic vesicles or bound to various metal binding proteins, including metallothioneins and S100β (Aschner *et al.*, 1997; Masters *et al.*, 1994; Brouwer, 1990; Heizmann and Cox, 1998; Cheng and Reynolds, 1998; Choi and Koh, 1998). The zinc-induced development of amyloid fibrillogenesis most likely requires the liberation of zinc at the site of activity.

Astrocytic proteins, such as S100β and metallothioneins I and II, are also major sources of zinc (Cheng and Reynolds, 1998; Frederickson, 1989) and are known to be labile. While metallothionein I and II are not altered in the AD state, S100β has been shown to be increased dramatically in Alzheimer's disease (Griffin *et al.*, 1989). While S100β is more closely associated with calcium binding, it has the potential to bind, and provide a source of zinc. Another metallothionein, metallothionein III (MT III), is believed to be produced by neurons and is another localized source of zinc. MT III is reduced in AD (Uchida *et al.*, 1991; Yu *et al.*, in press). The facilitation of zinc transport to the appropriate site of activity is an important element in understanding the development of amyloid fibrillogenesis and the role zinc plays in this process. The neuroprotective element of zinc binding proteins provides insights into neuronal biochemistry and also represents a potential target for therapeutic intervention.

4.3 EXPERIMENTAL PROCEDURES

4.3.1 Aβ Peptide Synthesis and Protein Purifications

Amyloid-β residues 1-40 (Aβ40) was synthesized by solid phase Fmoc-chemistry by the Hospital for Sick Children's Biotechnology Centre (Toronto, ON). Peptides were purified by reverse phase HPLC on a C18μ bondpak column. Peptides were initially dissolved in 0.5 mL of 100% trifluoroacetic acid (TFA, Aldrich Chemicals, Milwaukee, WI), diluted in distilled H_2O, immediately lyophilized and stored at -20 °C until use. Purified synthetic Aβ was allowed to incubate for 24 hours at 37°C prior to addition to culture.

A protein extract containing S100β was made from fresh bovine brains using a method described by Yu and Fraser (2001). For MT-III, fresh bovine brain tissue was homogenized in a 10% solution using 20mM Tris (pH= 8.0). The sample was frozen and thawed three times, sonicated for 3 x 15 seconds and centrifuged at 5000 X g for 30 minutes. The supernatant was collected and saturated with copper and zinc, heated at 100°C for 1 min and left to cool on ice. The sample was centrifuged again at 14 000 x g for 15 minutes. The supernatant was further purified by HPLC using a C18 bondpak column.

4.3.2 PC-12 Cultures and Treatment with Zinc and Aβ

PC-12 cells were maintained at 37°C under 5% CO_2 using 10% fetal bovine serum (FBS) in Dulbecco's modified eagle's medium (DMEM) (Gibco BRL). One thousand PC-12 cells were added per well on a 96 well plate previously treated with poly-L-lysine (Sigma, St. Louis) and differentiated using grade I 2.5S nerve growth factor (NGF, Alomone Labs, Jerusalem) applied daily to PC-12 cell cultures at a concentration of 1ng/mL for four days.

4.3.3 Effects of zinc, S100β, MT III and β-amyloid (Aβ) in PC-12 cells

NGF-differentiated PC-12 cells were incubated using concentrations of zinc sulphate (pH=7.4) ranging from 0.1μM to 1mM; S100β or MT III at concentrations of 10ng/mL to 100μg/mL (n=8); or Aβ using four concentrations, 1μM, 5μM, 10μM and 20μM (n=4).

4.3.4 Effect of amyloid and zinc on Differentiated PC 12 cell culture

Treatment of PC-12 cells with zinc was repeated again with the concomitant addition of 1 and 10μM Aβ. Samples were repeated four times and the culture was maintained for 24 hours following protein and metal addition.

4.3.5 Effect of Metal Binding Proteins MT III and S100β in Aβ/zinc Treated Differentiated PC-12 Cultures

Upon establishing the toxicity profile of differentiated PC-12 cells in cultures treated with Aβ and zinc, the metal binding proteins S100β or metallothionein III were added simultaneously to 10μM Aβ + 10μM zinc addition. An SRB assay was performed to assess any additive or subtractive effect of the addition of MT III or S100β.

4.3.6 Evaluation of Cell Viability - Sulforhodamine B (SRB) assay

SRB is a dye that binds to basic proteins on the surface of living cells and is a marker for viable cells where dyes that are incorporated internally are not appropriate. After 24 hours of incubation, the cells were fixed using 15% trichloroacetic acid (TCA) in distilled water for 30 minutes at 4°C. The plate was washed with distilled water and dried. Cells

were exposed to 40mg/mL of SRB in 1% acetic acid / distilled water was applied to each sample and let stand for 2 hours at room temperature. Upon incubation, the plate was washed three times with 1% acetic acid to remove non-specific SRB binding. Samples were read following the addition of 100μL of 100mM unbuffered Tris-base using a 450nm filter on the Biorad Ultramark plate reader.

4.3.7 Statistical Analysis and Determination of LC_{50}

Mean ± 1 standard deviation (SD) is graphically displayed for each treatment group. In cases where one test group is compared to another, a Student's unpaired T-test was used as the statistical test, where $p<0.05$ was deemed statistically significant. In determining toxicity, the final concentration point at which there is 50% response when compared to control is deemed the point at which there is 50% of maximal toxicity to the cells (LC_{50}).

4.3.8 Confocal Microscopy Analysis of Aβ Association with Cell Membranes

PC-12 cells were placed at low density on poly-L-lysine coated glass coverslips and differentiated with NGF for four days. The following protein/metal combinations were added to culture: a) 10μM Aβ alone; b) 10μM Aβ + 10μM zinc; c) 10μM Aβ + 10μM zinc + 10μg/mL S100β, and; d) 10μM Aβ+ 10μM zinc + 10μg/mL MT III.

After 24 hours, the cells were washed with fresh medium and fixed using 2% paraformaldehyde for 30 minutes, washed with PBS and stained for Aβ. PC-12 cells were stained by immunofluorescence using Aβ monoclonal antibody (clone 4G8; Senetek, St. Louis) and pan cadherin (1:250, Sigma), a cell membrane protein. Cells were visualized using a Nikon TE300 inverted microscope attached to a Biorad Radiance 2000 confocal

microscope. Samples were viewed under sequential transmission of red/green emission (488/568nm excitation respectively with 515-530/600-640nm emission) and 0.5μm sections over a range of 4μm were analyzed.

4.4 RESULTS

In order to establish a baseline for this study, we examined the individual effects of zinc, amyloid and the metal binding proteins. In the SRB assay for amyloid toxicity, we observed a dose-response activity, where the half-maximal lethal concentration (LC_{50}) is 10μM $A\beta_{(1-40)}$ (Fig. 1A). In our analysis, there was no statistical reduction in cell viability between the non-treated and 1μM Aβ level. The maximal toxicity was achieved at levels beyond 20μM, which itself resulted in only 20.0±4.7% of the cells remaining viable.

Zinc induced toxicity in neuronal cells has been previously characterized (Yokoyama *et al.*, 1986). In this study the LC_{50} for zinc, from zinc sulphate was 87.5μM (Fig. 4.1B). Other studies have found similar toxicity values for zinc within PC-12 cells (Kozumi *et al.*, 1995; Wang *et al.*, 1999). When 1μM Aβ was added to culture with zinc, there was no combined effect in the degree of toxicity as compared to no Aβ treatment. This corresponds to the results from treatment with 1μM amyloid alone, suggesting that at low concentrations of Aβ, the effect of toxicity is derived from zinc alone. The LC_{50} for zinc with 1μM Aβ is the same as zinc alone, (*i.e.*, 87.5μM). There is a significant difference in response found in the co-addition of 10μM Aβ with zinc. There is a synergistic effect whereby the toxicity curve has shifted downward, resulting in an LC_{50} in this system of 20μM zinc (Fig. 4.1b). Furthermore, where the LC_{50} of Aβ toxicity is 10μM, the percentage of viable cells is reduced beyond this amount upon zinc addition, suggesting a common toxic pathway.

As zinc in the nervous system is rarely in the free ionic or chemical form physiologically, we assessed if metal binding proteins altered the toxicity of Aβ. S100β was examined due to its zinc binding properties and due to an increased expression level in

Alzheimer's disease. Preliminary studies examining the effect of S100β alone found that this protein was toxic only at high concentrations. There was a significant toxic effect of S100β at levels greater than 100μg/mL (approximately 10μM), with an LC_{50} at 180μg/mL (Fig. 4.2). There is a clear additive effect when both S100β and Aβ were co-administered to culture. With the addition of 10μM Aβ, the LC_{50} for S100β was reduced to between 1 and 7.5μg/mL (Fig. 4.2). S100β can bind up to 5 zinc ions per molecule (Donato, 1991) and may facilitate zinc induced toxicity of Aβ more efficiently by acting as a metal donor.

Another zinc binding protein found in the central nervous system is metallothionein-III. Unlike its other family members, MT-III is localized to the brain and MT-III is decreased in Alzheimer's disease whereas MT-I and -II are not (Uchida *et al.*, 1991; Yu et al., 2001). In our study, we observed the characteristic reduction in neurite development and axonal retraction upon the addition of MT-III. There was a reduction in the cellular measurement since the SRB assay is measured by the amount of cell surface protein binding of living cells, and due to MT-III induced neurite retraction, this would decline in a concentration-dependent manner (Fig. 4.3). In contrast to S100β, MT-III at concentrations as high as 10μg/mL did not alter the viability of PC-12 cells treated with amyloid (Fig. 4.3). As with 10μM Aβ alone, MT-III addition to did not alter the survival profile. The lack of protective or detrimental effect is possibly a consequence of Aβ fibril formation occurring regardless of zinc enhancement.

Consistent with the toxicity findings, we observed relevant cell surface aggregation of Aβ on NGF-differentiated PC-12 cells. For this study, we used non-aged Aβ to allow real time aggregation of Aβ onto the cells starting with monomer form in order to observe the effect of zinc or metal binding proteins on the rate of aggregation. Within the 24-hour

incubation period, Aβ alone formed small aggregates primarily on the axons and dendrites of the cells (Fig. 4.4A). With the addition of 10μM zinc, a concentration that was not toxic in the previous studies, the deposition of Aβ was increased although the aggregates remained primarily on the cell surface of the neurites (Fig. 4.4B). The addition of S100β resulted in a significantly greater degree of Aβ deposition (Fig. 4C). Furthermore with zinc addition, this deposition was increased on the cell body, a pattern not observed in Aβ and Aβ+zinc treatments (Fig. 4.4D). In addition to the increased rate and distribution of Aβ deposition, fewer viable cells were observed. In the previous examination of S100β with Aβ (LC_{50} = 7.5μM), whereas we used 10μM of Aβ in this assay. The dramatic decline in the number of cells may account for the increased proportion of Aβ binding to cells.

Finally, the coincubation of 10μg/mL MT-III with Aβ yielded a decrease in Aβ deposition on the neurites (Fig. 4.4E), with little observed aggregation on the cell body as was noted with the S100β treatment. This suggests that MT-III does not readily release its bound zinc. Upon closer examination, Aβ deposition appeared to be decreased when compared to Aβ+zinc and Aβ+zinc+S100β samples. This deposition was closer in magnitude to that observed in the Aβ alone sample (Fig. 4.4A), suggesting that zinc induction of Aβ aggregation and deposition is limited by the presence of MT-III. The addition of zinc to Aβ + MT-III treated cells did not alter the amyloid-beta deposition when compared to other conditions (Fig. 4.4F). This suggests that MT-III may act as a regulatory mechanism for zinc to prevent metal release and subsequent enhancement of Aβ aggregation.

4.5 DISCUSSION

As various studies have demonstrated, Aβ fibril formation *in vivo* is enhanced by zinc ions (Bush *et al.*, 1994a; Esker *et al.*, 1996; Yang *et al.*, 2000; Bush *et al.*, 1994b). The current study was designed to investigate the role of zinc on fibrillogenesis and aggregation as it related to metal-binding proteins, MT-III or S100β. Zinc may not be unique in terms of altering Aβ aggregation, as other divalent cations such as copper, iron and aluminum can induce similar precipitation of the peptide (Bush *et al.*, 1994a; Mantyh *et al.*, 1993; Lovell *et al.*, 1998; Cornett *et al.*, 1998; Atwood *et al.*, 1998). However, calcium does not have any effect on amyloid aggregation (Mantyh *et al.*, 1993; Esler *et al.*, 1996). The objective in this study was not to explore *in vitro*, the consequences of alterations in specific metal binding proteins related to AD. As MT-III and S100β expression levels are altered in Alzheimer's disease, they may alter zinc homeostasis and their effects on Aβ aggregation may be an important contributing factor.

Zinc is the second most abundant essential ion in the human system (Vallee and Flachuk, 1993) and is involved in over 200 enzymes and catalytic processes Hartley *et al.*, 1999; Vallee and Auld, 1992; Rink and Gabriel, 2000; Wu and Wu, 1987; Wu *et al.*, 1992; Hooper *et al.*, 2000). In AD, zinc homeostasis, specifically uptake and distribution, is disrupted. Furthermore, in some brain regions, such as the hippocampal and amygdala regions, zinc levels are increased (Hartley *et al.*, 1999; Christensen and Frederickson, 1998; Wang *et al.*, 1999; Cuajungco and Lees, 1998; Corrigan *et al.*, 1993; Danscher *et al.*, 1997; Deibel *et al.*, 1996; Samudralwar *et al.*, 1995; Thompson *et al.*, 2000). Zinc is also elevated in Aβ deposits at millimolar concentrations (Lovell *et al.*, 1998, Suh *et al.*, 2000a, Ivins *et*

al., 1999). Zinc within a cell is normally well regulated (Colvin, 1998; Colvin *et al.*, 2000; Cheng and Reynolds, 1998; Frederickson, 1989), with very low internal concentrations of zinc causing cell death (Choi and Koh, 1989; Ivins *et al.*, 1997). Within the cell, it is predominantly sequestered in proteins and vesicles, such as zinc transporters and metallothioneins.

Previous studies examining the effect of zinc on Aβ aggregation have shown a dramatic increase in Aβ fibrillization and β-sheet conformation, as well as increased aggregation of the peptide. This work examined the *in vitro* phenomenon using NGF-differentiated PC-12 cells to emulate a neuronal system. In agreement with other groups, we observed that Aβ is toxic in a concentration dependent mode (Burdick *et al.*, 1992). For example, the half maximal (LC_{50}) concentration was slightly higher than 10μM, a figure observed by others (Ivins *et al.*, 1999; Fukuyama *et al.*, 1994). Aβ toxicity in PC-12 cells was enhanced by the addition of small amounts of zinc (10μM). What is intriguing is that the amount of zinc required to increase PC-12 toxicity response to Aβ is now less than 10μM with Aβ, whereas without Aβ, the figure is approximately 90μM.

Metal binding proteins provide and sequester the pool of critical ions involved in many cellular functions. The dysregulation of this homeostasis can result in altered ion fluxes and may contribute to the degenerative process we observe with Aβ aggregation and its effect on neuronal cells. To evaluate whether there is a meaningful correlation in altered expression of metal binding proteins and their effects in Alzheimer's disease with respect to Aβ, we examined S100β and MT-III. In Alzheimer's disease, they both have altered expression levels. S100β is dramatically increased in aging, as well as in AD (Griffin *et al.*, 1989). MT-III is decreased in some regions of the brain, which coincide with these areas

susceptible to deterioration in AD (Uchida *et al.*, 1991; Yu *et al.*, 2001). Both proteins are predominantly CNS-specific and also bind zinc.

From our observations, S100β appears to potentiate Aβ aggregation. The effect is similar to PC-12 cells treated with Aβ and zinc. The addition of purified bovine S100β to culture resulted in a dose dependent cytotoxic action on cells coincubated with zinc and Aβ. This was confirmed by confocal microscopy, which showed that S100β induced Aβ deposition onto the cells in a manner similar to zinc treatment. This data suggests that S100β may act as donor or shuttle to provide zinc to Aβ to enhance its aggregation. The addition of both zinc and S100β acted in a synergistic fashion producing the greatest degree of Aβ deposition out of all the experimental conditions.

In contrast to S100β, MT-III appears to act as a reservoir that removes free zinc ions and prevents their participation in Aβ aggregation. MT-III failed to protect the PC-12 cells from fibrillar peptide but it also did not enhance it. The possible scenario would be that the peptide that is already aggregated will affect the cells, but without additional enhancers, such as zinc, the activity and toxicity will not be increased. Using confocal microscopy, we observed the addition of fresh (non-aged) peptide and found that there is the same degree of aggregation and deposition on the cells as with Aβ addition alone. This would represent the proportion of Aβ aggregation that occurs independent of zinc function.

This work was performed to examine the endogenous function and the consequence of reduced MT-III and increased S100β and how this relates to the Alzheimer's disease process. Potentially, an increase in S100β, coupled with its enhancement of Aβ aggregation, alongside the loss of metallothionein III activity, which inhibits zinc-induced Aβ aggregation, will result in a synergistic increase in Aβ aggregation and deposition on cells.

The net effect of this is that alterations in metal-binding proteins in AD contribute to the aggregation of Aβ.

Elevated zinc levels in Alzheimer's brain tissue alterations can be attributed to changes in its regulation. One pathological consequence is the enhancement of Aβ aggregation. An integral component in maintaining cellular zinc levels is the appropriate sequestration, utilization and turnover of the metal ion. Zinc is an abundant and essential metal to biological function and alterations in its free form are potentially toxic. In this case, it is not only the individual effect of zinc, but also its ability to aggregate Aβ into a toxic substrate. This study demonstrates the effect of alteration in two zinc-binding components, S100β and MT-III, and how it can also contribute to zinc induction of Aβ aggregation. Current studies into zinc and copper induction of Aβ aggregation and deposition use metal chelators to reduce the ionic load (Atwood *et al.*, 2000; Cherny *et al.*, 2000). This study provides endogenous substrates to examine the role metals can play in Aβ aggregation and the *in vitro* effect on cells and finds that there is an important role of S100β and MT-III in regulating zinc homeostasis and the potential downstream consequences of its dysregulation.

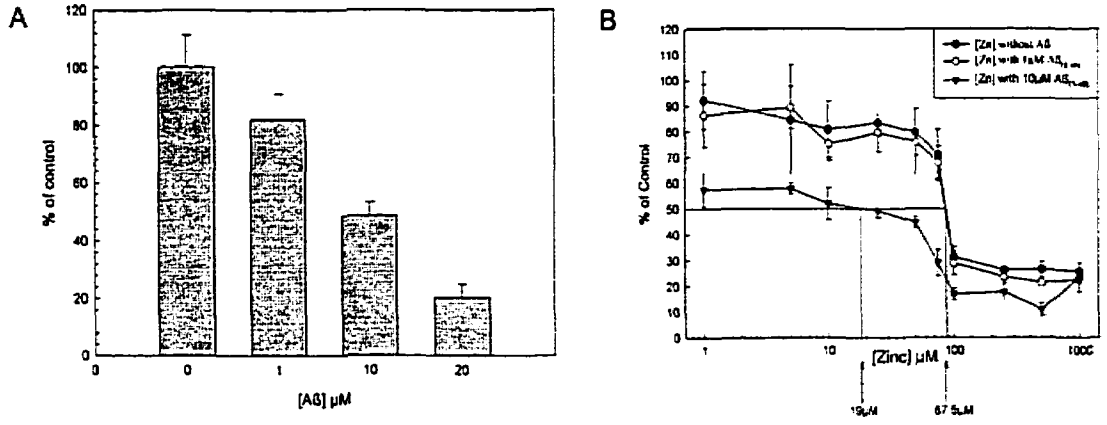

Figure 4.1: Aβ (**A**), zinc alone and with Aβ (**B**) toxicity assays were performed to establish a baseline effect of individual treatments. **A** - Sulforhodamine B assay for NGF differentiated PC-12 cells treated Aβ for a 24 hour period. Aβ was permitted to form fibrils over 24 hours prior to addition into the cell medium. Control and 1μM Aβ show no significant difference, whereas 10μM represents half control response and 20μM addition results in maximal loss of cellular activity. **B** - Comparison of cells treated with zinc, zinc + 1μM Aβ, and zinc + 10μM. PC-12 cells that were treated with zinc alone or zinc + 1μM Aβ did not show significantly different toxicity profiles. The LC_{50} for both is approximately 87.5μM zinc. Zinc + 10μM Aβ was approximately half of the control or 1μM Aβ response initially and at all concentrations of zinc had fewer viable cells than the control samples.

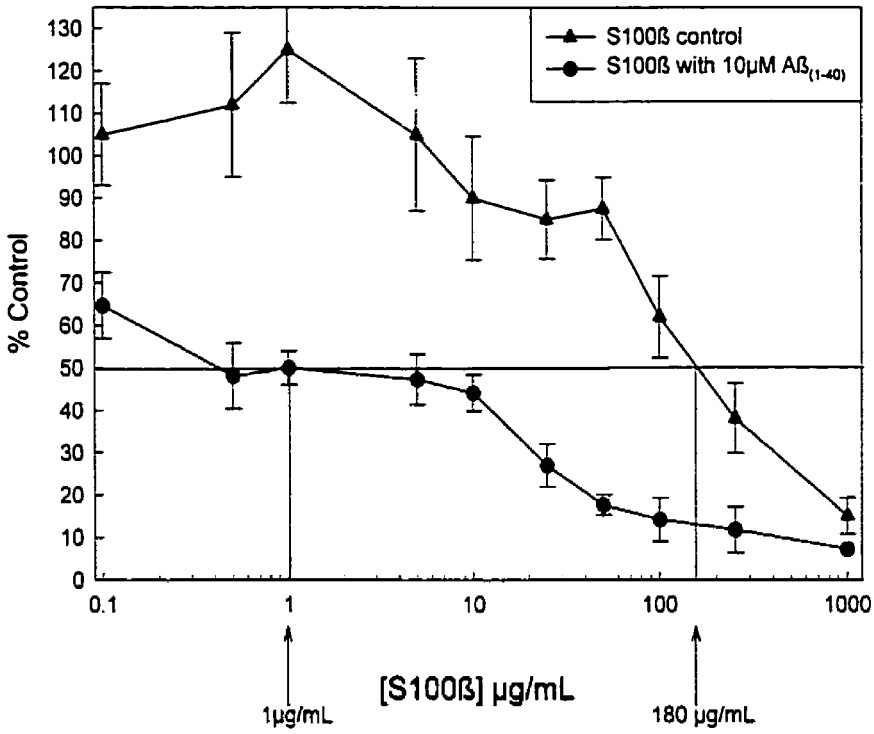

Figure 4.2: Effect of S100β alone and with Aβ. S100β is a neurotrophic factor and there is an associated increase in cellular activity. At higher concentrations, however, S100β appears toxic to the cells. The addition of 10µM Aβ appears to reduce the cellular viability. The Aβ toxicity effect is enhanced beginning at 10µg/mL S100β and by 50µg/mL S100β, the toxicity is similar to that maximally produced by 20µM Aβ alone.

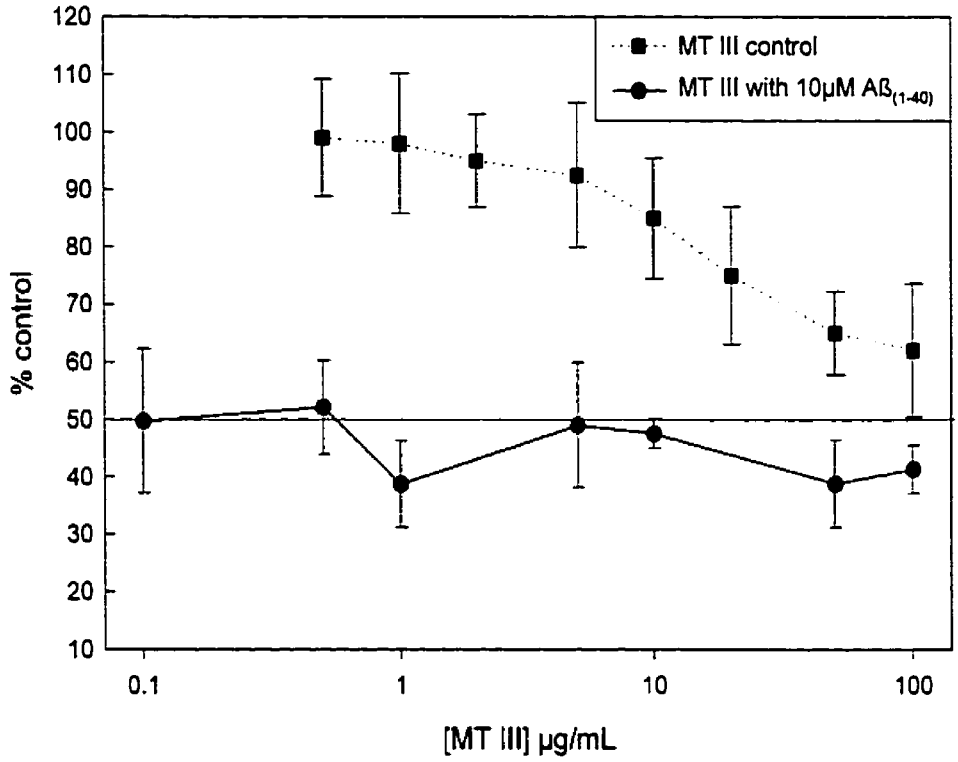

Figure 4.3: Effect of MT-III alone and with Aβ. MT-III alone did not alter PC-12 response significantly. There was also no change from the effect of Aβ+MT-III versus Aβ alone. 10μM Aβ reduced the cellular response to approximately 50%, whereas MT-III addition up to 50μg/mL did not significantly increase, or decrease the toxicity of Aβ.

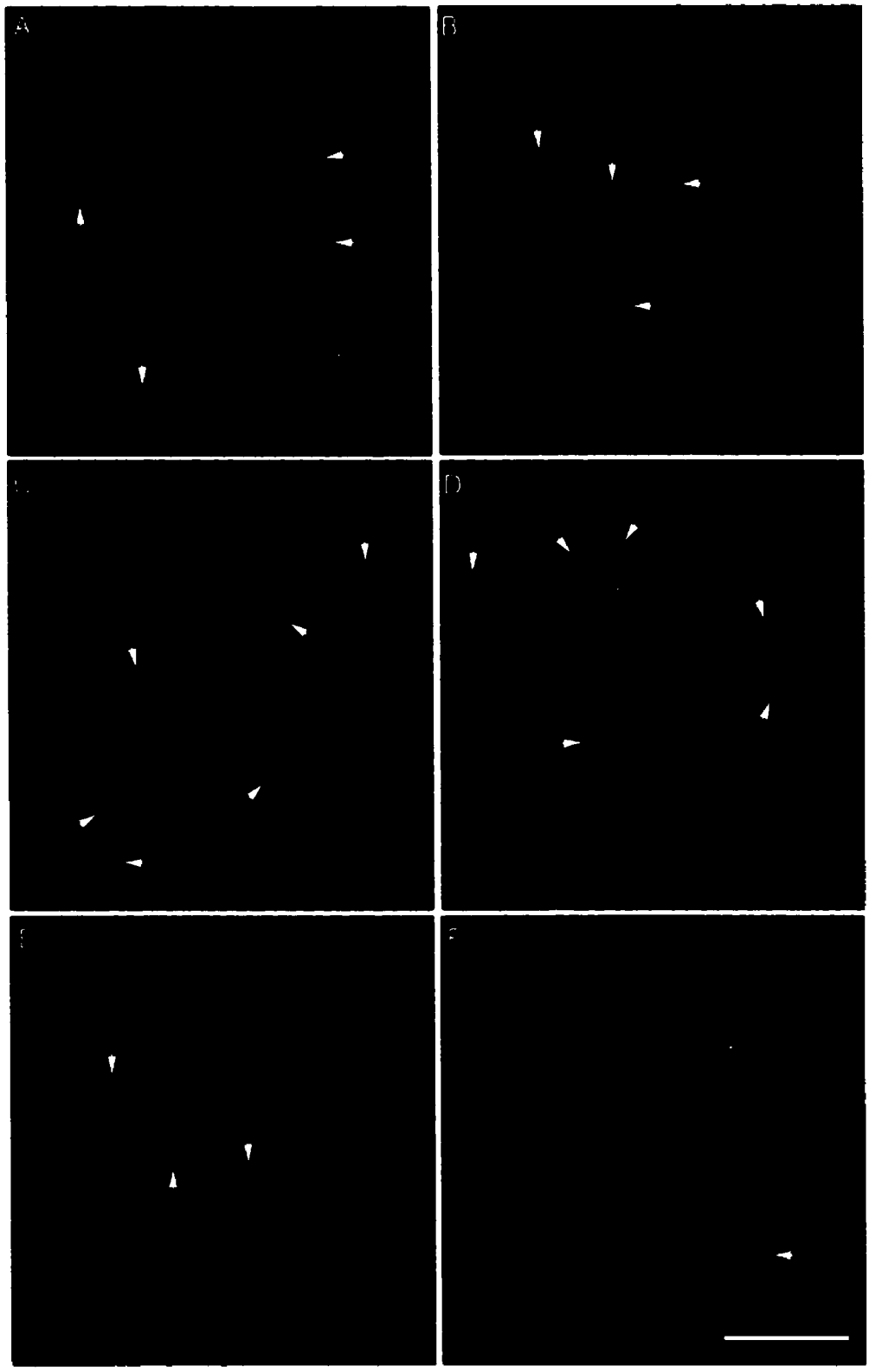
A
B
C
D
E
F

Figure 4.4: Laser confocal microscopy analysis of Aβ and zinc treatment in PC-12 cells alone and in the presence of metal-binding proteins S100β and MT-III. In Panel A, PC-12 cells were treated with 10 μM Aβ and allowed to incubate for 24 hours at 37°C. The PC-12 cells are labelled with a pan-cadherin stain (green fluorescence) and Aβ is indicated by red fluorescence emission. Arrows are used to indicate colocalization pattern of cell surface and Aβ aggregation. Under control conditions, there is some deposition of Aβ on the neuritic processes. The addition of zinc increased the amount deposited and there is also a greater degree on the cell body itself (Panel B). S100β increased the amount deposited on the cells in both Aβ alone (Panel C) and Aβ+zinc (Panel D). The addition of MT-III to Aβ alone and Aβ+zinc treated cells did not increase the deposition of Aβ to any degree greater than that observed in the control (Panel A) sample. 10μ is indicated by scalebar in Panel F.

Chapter 5

Authors - Wai Haung Yu and Paul E. Fraser

Title: S100β Interaction with Tau is Promoted by Zinc and Inhibited by Hyperphosphorylation in Alzheimer's Disease

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Contribution to Paper –

1) Designed and performed all experiments
2) Wrote and edited manuscript

Significance of Article to Thesis –

This paper examines S100β binding to proteins involved in Alzheimer's disease. It also discovers the relationship between neuritic sprouting observed in AD and how S100β is intimately involved via direct interaction to tau. Furthermore, this study notes that these events are aided by zinc binding and occur prior to phosphorylation of tau, a major entry point for the development of neurofibrillary tangles.

5.1 ABSTRACT

The zinc-binding protein, S100β, has been identified as an interacting partner with the microtubule associated protein, tau. Both proteins are individually affected in Alzheimer's disease (AD). S100β, is overexpressed in the disease, while hyperphosphorylated tau constitutes the primary component of neurofibrillary tangles. In this study, we examine factors that modulate their binding and the potential role the complex may play in AD pathogenesis. Zinc was identified as a critical component in the binding process and a primary modulator of S100β-associated cellular responses. Abnormally phosphorylated tau extracted from AD tissue displayed a dramatically reduced capacity to bind S100β, which was restored by pre-treatment with alkaline phosphatase. In differentiated SH-SY5Y cells, exogenous S100β was internalized and colocalized with tau consistent with an intracellular association. This was enhanced by the addition of zinc and eliminated by divalent metal chelators. S100β uptake was also accompanied by extensive neurite outgrowth that may be mediated by its interaction with tau. S100β-tau binding may represent a key pathway for neurite development possibly through S100β modulation of tau phosphorylation and/or functional stabilization of microtubules and process formation. S100β-tau interaction may be disrupted by hyperphosphorylation and/or imbalances in zinc metabolism and this may contribute to the neurite dystrophy associated with AD.

5.2 INTRODUCTION

S100β is a small molecular weight (10kD) zinc/calcium binding protein produced by astrocytes (Donato, 1991; Mrak *et al.*, 1995). In addition to metal binding, S100β has several functions that include a role in the cytokine cycle, inhibition of selected phosphokinases, including phosphokinase C (PKC), and the stimulation of neurite outgrowth (Griffin *et al.*, 1998; Kligman and Marshak, 1985; Heizmann *et al.* 1998; Baudier and Cole, 1988; Zimmer *et al.*, 1995; Marshak and Pena 1992). S100β is located on chromosome 21 and is increased in Down's syndrome and Alzheimer's disease (by as much as 20-fold) (Griffin *et al.*, 1989; Griffin *et al.*, 1998; Marshak *et al.*, 1992; Castets *et al.*, 1997). In AD, the pathology is defined by senile plaques and neurofibrillary tangles (NFT) that are accompanied by neuronal loss and aberrant neuritic sprouting (Masliah *et al.*, 1991). The neuritic response may be induced by the loss of neuronal connections or a cellular reaction to Aβ deposition (Mrak *et al.*, 1996). S100β overexpression in AD has been directly correlated with plaque-associated dystrophic neurite development and astrocyte activation, as well as S100β overproduction, may be a direct effect of the loss of neuronal connections and Aβ deposition (Van Eldik and Griffin, 1994; Mrak *et al.*, 1996; Sheng *et al.*, 2000). S100β levels are elevated in brain regions with a direct relationship to the presence of neuritic plaques (Sheng *et al.*, 1994). In addition, astrocyte activation and S100β expression may also be correlated with neurofibrillary tangle formation in AD (Sheng *et al.*, 1994)

This study examines the relationship between tau and S100β based on the observation that they are cellular binding partners and each may therefore regulate specific neurite outgrowth or tau hyperphosphorylation activity (Baudier and Cole, 1988; Sorci *et al.*, 2000).

Secondly, tau is a unique neuronal component that stabilizes microtubules leading to the formation of axonal processes and, in its hyperphosphorylated state, tau is the major component of neurofibrillary tangles (Ikura *et al.*, 1998; Mailliot *et al.*, 1998; Su *et al.*, 1994; Nagy *et al.*, 1995; Su *et al.*, 1994). Finally, although the mechanism is unknown, S100β can induce a similar neurite outgrowth that may be related to its association with tau. S100β has been shown to directly affect tau, for example, by its ability to block PKC phosphorylation at specific sites (Ser 262 and 313) (Singh *et al.*, 1996a; Lin *et al.*, 1994; Corracas, *et al.*, 1992). This activity may have a direct consequence for AD since loss of PKC phosphorylation increases the susceptibility of tau to hyperphosphorylation by GSK-3β (Singh *et al.*, 1996b; Tsujo *et al.*, 2000). This AD-related phosphorylation is considered to be a major factor in tau deposition and neurofibrillary degeneration (Ikura *et al.*, 1998; Mailliot *et al.*, 1998; Friedhoff *et al.*, 1998; Su *et al.*, 1994).

We have examined S100β binding proteins by affinity chromatography and immunoprecipitation to survey the potential involvement of other AD-associated proteins. In addition to tau, S100β binding to the amyloid precursor protein (APP), the Aβ peptide and the presenilins (PS1 & PS2) were also assessed. Among the proteins we evaluated, tau was the only significant binding protein and furthermore, based on immunofluorescence studies, co-localized with S100β upon internalization by neuronal cells. Zinc has also been implicated in some aspects of AD pathology, such as promotion of Aβ fibril formation (Bush *et al.*, 1994) and, when examined in the current system, it significantly affected the relationship between S100β and tau. This may be due to zinc-induced conformational changes that results in the exposure of a hydrophobic domain and could represent a key site for tau binding (Fujii *et al.*, 1986; Baudier and Cole, 1988; Baudier *et al.*, 1992). In addition,

changes to tau also regulated this interaction as shown by the altered binding of S100β to the AD-related hyperphosphorylated NFT-tau. Based upon our observations, S100β-tau binding, overexpression of S100β, and tau hyperphosphorylation in Alzheimer's disease pathology suggest that S100β-tau interaction may contribute to neuronal sprouting as well as neuronal dysfunction by increasing the neuron's exposure to toxic elements and potentially lead to neurofibrillary tangle generation.

5.3 METHODS

5.3.1 Purification of S100β

Extracts containing S100β were prepared from fresh bovine brains using the method described by Isobe *et al.* (1977). A 20% homogenate was made in a potassium phosphate buffer (0.1M KPO_4, pH=7.1, 1mM EDTA, 1μg/mL aprotinin, 1μg/mL leupeptin and 1mM polymethonyl sulfate (PMSF)) with 2.66M (or 50%) ammonium sulfate. Cell debris was removed by centrifugation at 10, 000 X g and the supernatant adjusted to 85% $AmSO_4$ at pH 4.2 and incubated at 4°C for 2 hr. Precipitated proteins were recovered by centrifugation, dialyzed against phosphate buffer and stored at –20°C in lyophilized form. From this crude material, S100β was purified using a modified method as described by Baudier *et al.* (1982). Crude extracts were dissolved in the elution buffer (50mM Tris-Base, pH=7.4) with 1mM $ZnSO_4$ and applied to a Phenyl Sepharose 650M column (ToyoPearl, Montgomeryville, PA). S100β was eluted using a step gradient containing 300mM NaCl, 0.25mM $ZnSO_4$ or 2mM EDTA. Protein purity was assessed by SDS-PAGE with Coomasie staining and by western blotting with an S100β monoclonal antibody (clone SH-B1; Sigma, St. Louis).

5.3.2 Gel Electrophoresis and Western Blotting

S100β (1μg) was dissolved in Laemmli buffer and separated on a 10-20% Tricine gel (Novex, Carlsbad, CA). Gels were either stained with 0.2% CBR in 5% acetic acid, or transferred to a polyvinyl difluoride (PVDF) membrane. The membrane was washed in Tris buffered saline (200mM Tris-base, pH=7.4, 150mM NaCl; TBS), blocked with skim milk and incubated overnight with the required antibody. Immunoreactive bands were identified

with HRP-conjugated secondary antibodies and visualized using enhanced chemiluminescence (ECL; Amersham) with film exposure.

5.3.3 S100β affinity chromatography and identification of binding proteins

Purified S100β was immobilized on AffiGel-10 (BioRad) and equilibrated in 100mM HEPES with 0.25mM $ZnSO_4$. Immobilized S100β was incubated with a human brain tissue homogenate (10% wt/vol) and non-specific binding proteins were removed by washing with the initial buffer. A high salt (100mM HEPES, 1M NaCl, 0.25mM $ZnSO_4$) wash was used to elute proteins with weak S100β interactions. Zinc-dependent binding proteins were subsequently eluted with 1mM EDTA and any remaining bound elements were removed with 1M urea. All samples were collected and dialyzed then stored at –20°C in their lyophilized form. Eluted proteins were analyzed on 4-20% Tricine gels (Novex) and examined by silver staining and by western blotting. Antibodies corresponding to S100β, Aβ (clone 6F/3D, DAKO), tau (DAKO) and a presenilin antisera (Yu *et al.*, 1998) were used to determine if they were capable of binding to S100β.

5.3.4 Formation of S100β complexes with normal and AD tau

AD and control brain were homogenized (10% w/v) in 0.1M KH_2PO_4, 2mM EDTA, 2mM EGTA, and protease inhibitors. Samples were centrifuged for 45 minutes at 20 000 x g and the supernatant was fractionated using 35% and 55% ammonium sulfate to produce a tau enriched fraction. Crude protein precipitates were resuspended in 20mM Tris and 0.5M NaCl, pH 7.6 with protease inhibitors. Samples were boiled, centrifuges at 25 000 X g for 30 minutes and control aliquots were collected. To assess the effects of phosphorylation on

S100β binding, samples were also treated with alkaline phosphatase (Sigma) for 30 minutes at 37°C. Binding of S100β with tau from these enriched samples was assessed by immunoprecipitation. Aliquots of the brain extracts (50μg total protein) were combined with 1μg purified bovine S100β and 10μl of S100β monoclonal antibody. The mixture was incubated overnight at 4°C and the S100β-containing complexes were recovered by immunoprecipitation by protein-G sepharose. Beads were washed with buffer containing 50mM Tris with 150mM NaCl and 0.5% NP-40, and S100β with bound proteins eluted with 500mM NaCl with 1mM EDTA. Samples were collected, dialyzed and examined by western blotting using tau antibodies.

5.3.5 S100β internalization and subcellular distribution

Bovine S100β (final concentration = 5μg/mL) was added to culture and incubated for pulse of 4 or 24 hours. Cells were washed with fresh medium and harvested at 0, 15, 30, 60 minutes and 4, 24, 48 hours. Cells lysates were examined by immunoblotting to determine cellular uptake of S100β. SH-SY5Y cells were grown in 10% fetal bovine serum / DMEM (Gibco) at 37°C under 5% CO_2. Cells were placed on poly-L-lysine coated coverslips and differentiated using 10μM trans-retinoic acid. To examine co-localization with tau, S100β was pre-incubated with the cells for 4, 12 and 24 hours under control conditions or with 50μM EDTA or 5μM EGTA for 1hour prior to addition of S100β or with 10μg/mL $ZnSO_4$. Cells were fixed with 2% paraformaldehyde and examined by immunofluorescence using a Nikon TE300 inverted microscope attached to a Biorad Radiance 2000 laser confocal system.

5.4 RESULTS

5.4.1 Identification and analysis of S100β binding proteins

Interactions of brain derived proteins, such as tau, were initially examined by affinity chromatography using immobilized S100β as the primary substrate. A native S100β secondary structure was maintained in the presence of calcium and zinc to obtain physiologically relevant conditions for the evaluation of binding proteins (Baudier *et al.*, 1982). A series of increasing elution stringencies were used to determine the relative affinities of S100β binding proteins. Proteins that failed to bind to the S100β substrate were recovered in the initial wash. This was followed by a high salt elution to isolate proteins with weak ionic binding properties. High affinity S100β-associated proteins were removed by the addition of zinc chelators, which caused a structural rearrangement of S100β. Previous studies have shown that zinc exposes a hydrophobic domain, which represents a potential binding site for its cellular partners (Isobe *et al.*, 1977). Finally, any remaining proteins bound to the affinity column were removed with a denaturing urea wash and each of these fractions was examined by direct silver staining as well as western blotting.

Immunoblotting of the various elutions demonstrated that tau constituted a principal S100β binding protein. All other AD-related proteins such as APP, Aβ, PS1 and PS2 did not show any significant S100β binding and were recovered in the initial elution. Tau binding was particularly evident in the samples obtained from control cases where strong signals were observed for all brain regions (Fig. 1A). The control tau was only eluted upon zinc chelation with EDTA suggesting that the observed conformation changes are important for binding. In contrast, in the comparable elutions, there was a marked decrease in the amount

from the AD tau fraction (Fig. 5.1A). The lack of tau was not due to loss of immunoreactivity caused by changes in the AD-related protein as a polyclonal, non-phosphorylation-dependent antibody was used. To confirm this, additional antisera were used (*e.g.*, phosphorylation epitopes detected by the antibody AT8) which demonstrated a similar lack of tau binding. Examination of the complete range of elutions revealed that AD-tau was found in both the flow-through and salt washes. Based on this finding it was determined that tau from AD samples had a significantly lower affinity for S100β.

To examine potential changes in the S100β levels between AD and control cases, western blotting of comparable tissue samples was investigated. In the AD cases that showed the loss of tau binding to S100β, appreciable increases in the S100β levels were observed in all AD brain samples (Fig. 5.1B). The reason for the increased expression is unclear but does suggest an imbalance in S100β levels that may represent a compensatory mechanism for reduced activity. For example, if S100β does modulate tau function and/or metabolism then the loss of this interaction in AD may induce the elevated expression.

5.4.2 Identification of S100β and tau complex

In order to assess further the binding of S100β to tau, immunoprecipitation of *in vitro* complexes was examined using both AD and control extracted samples. To accomplish this, a tau-enriched fraction was obtained from the brain homogenates through ammonium sulphate precipitation and incubated with purified S100β. The effects of tau phosphorylation on S100β–tau binding were also examined by immunoprecipitation with untreated extracts as well as following incubation with alkaline phosphatase. Since AD-tau is heavily phosphorylated this may be one reason for the observed reduction in its binding to S100β.

Immunoprecipitation of untreated AD extracts using an anti-S100β antibody yielded very low or undetectable levels of associated tau in all tissues examined (Fig. 5.2). This finding is consistent with the affinity chromatography results and suggests an impaired binding. In contrast, similar immunoprecipitation control samples produced a robust level of binding of tau to S100β. The high level of tau immunoreactivity reflects the amount of binding to S100β in immunoprecipitation samples relative to the same amount of protein used in the AD samples. The formation of the S100β-tau complex in the control extracts was also zinc-dependent. This event was demonstrated by the removal of zinc with EDTA, followed by the subsequent release of tau from immunoprecipitated S100β. This observation is consistent with the elution profile from the affinity column which facilitated the removal of tau from the immobilized S100β. Dephosphorylation of tau by alkaline phosphatase restored the normal, possibly functional, binding of tau to S100β (Fig. 5.2). In all AD cases, we observed a significantly higher level of binding after tau dephosphorylation. There was little or no change in the amount of tau that could be immunoprecipitated in the comparable control samples following alkaline phosphatase treatment. Restoration of binding upon dephosphorylation of tau indicates a possible mechanism for the lack of S100β-tau interaction in the AD cases.

5.4.3 Internalization and subcellular distribution of S100β in neuronal cells

S100β has a stimulatory activity on neurite outgrowth which may result from metal influx (calcium), cytokine activation, activation of phosphokinases to initiate axonal growth or microtubule stabilization (Mrak *et al.*, 1996; Baudier *et al.*, 1987a, 1988, 1990;Sheng *et*

al., 1996; Sheu *et al.*, 1994; Lin *et al.*, 1994). One possibility that we explored was the direct uptake of exogenous S100β by neuronal cell lines and the effects of this internalization on tau. Purified S100β was added to cultures of SH-SY5Y cells and was pulsed for 4 or 24 hours and then removed from the medium. Examination of cell lysates for S100β indicated that following 4 hours incubation relatively low levels of the S100β dimer were observed (Fig. 5.3). When examined after different incubation times (4 and 24 hr), the amount of S100β slowly decreased with a significant reduction observed at 4 hrs and a complete loss of cell-associated protein at 24 hrs. Incubation for a 24 hr period resulted in substantially greater amounts of S100β in the cell lysates including both monomeric and dimeric forms (Fig. 5.3). These levels were maintained 4 hrs post-incubation and detectable, but at reduced levels and were not observed following a 24 hr clearance period. These observations indicate that significant quantities of S100β associate with the cells and are maintained over long periods of time.

It is unclear from the western blotting data whether the S100β merely accumulates via non-specific binding to the plasma membrane or if the cells are capable of internalizing the exogenous protein. To resolve this issue, retinoic acid differentiated SH-SY5Y cells were used to produce a neuronal-like phenotype and the distribution of S100β examined by immunofluorescence. Cells incubated with purified bovine S100β displayed modest amounts of intracellular S100β staining following exposures for 4 and 12 hrs (Fig. 5.4). Consistent with the western blotting data, substantial levels of S100β were found following the 24 hr incubation (Fig. 5.4D). S100β immunoreactivity was distributed within the cell body and extended into the processes but was absent from the nuclear region.

The degree of S100β internalization was also affected by zinc as shown by the increased level of staining within cells, as compared to control, when zinc was added to the medium and co-incubated for 24 hours (Fig. 5.5). The effect of zinc (and possibly other divalent metals) was supported by EDTA treatment that has a higher affinity for the metal as compared to S100β. Under these metal-depleted conditions, the level of S100β was markedly reduced in the SH-SY5Y cultures as compared to controls (Fig. 5.5C). To examine the effects of other divalent cations, the calcium specific chelator EGTA was added to our cultures in order to block free and extracellular calcium. Low EGTA concentrations were used as they were not toxic and do not block neuritic sprouting but were sufficient to bind a significant proportion of free calcium. EGTA-treated cells exhibited comparable S100β staining providing additional support for the specific role of zinc (data not shown). Cumulatively, the western blotting and immunofluorescence studies suggest that S100β is actively internalized by the cells as opposed to surface association. This uptake has a number of implications for the mechanism of S100β activity in neuronal systems and its possible relationship to tau function.

5.4.4 Co-localization of S100β with tau and enhanced neurite outgrowth

To investigate the relationship between S100β–tau binding and neurite outgrowth, differentiated SH-SY5Y cells were allowed to internalize S100β and its subcellular distribution with respect to tau was examined by immunofluorescence. Under control conditions, S100β was broadly distributed within the cell body and some processes. Furthermore, in the double-labelled cells, the staining overlaps to some degree with tau (Fig 5.6A). However, a zinc-induced increase in the level of S100β within the cell produced a

much more defined co-localization with tau. This is particularly evident within the processes where the S100β and tau coincided as punctate staining which was observed in virtually all neurites (Fig. 5.6B, arrows). Colocalization of S100β was also time-dependent as 24 hrs of incubation produced higher levels of overlapping signals when compared to the 4 or 12 hr samples. To ensure that there were no significant changes in tau, the S100β treated cells were also analyzed for changes in phosphorylation using the PHF-tau AT8 antibody. AT8 immunoreactivity was not detected in any of the treated cells, at any timepoints (data not shown). The effects of zinc and the enhanced co-localization may reflect simply an increased cellular uptake of S100β or metal binding may promote a preferred conformation that facilitates tau binding. This latter possibility would be consistent with our affinity chromatography and immunoprecipitation results. These findings suggest that internalized S100β may be associated with tau and thereby affect tau function and/or metabolic events such as phosphorylation.

Tau is one of the key elements that control axonal growth and may be modulated, to some degree, by interactions with S100β. This hypothesis is supported in our experimental system by the response of the SH-SY5Y cells to S100β and zinc. Even with retinoic acid differentiation, SH-SY5Y cells do not produce extensive process formation and have a predominantly 'spindle-type' morphology (Fig. 5.7A). With the addition of S100β, a greater number of neurites were observed when visualized using an antibody staining for cadherins on the cell-surface (Fig 5.7B). Neurite outgrowth was even more pronounced in the presence of zinc where enhanced S100β uptake resulted in increased number of neurites with extensive outgrowth that produced both longer networks of processes (Fig. 7C). Under these conditions, abnormal neuritic sprouting was also observed with processes emanating from the

cell body. Stimulation of neurites and co-localization of S100β with tau provides additional evidence for a physiological role for their interaction.

5.5 DISCUSSION

These studies were performed to establish the binding of S100β to tau and the chemical properties involved, as well as identify its relevance to Alzheimer's disease. Our findings demonstrate that S100β binds to tau. In addition, this interaction is enhanced by zinc and inhibited by tau hyperphosphorylation. The functional aspects of S100β-tau binding may impact on several different pathways that are regulated by the two proteins. For example, S100β may provide a scaffolding structure for tau to stabilize microtubules and possibly contribute to the abnormal neuritic dystrophy that is observed in AD (Tam, 1990; Baudier and Cole, 1988; Azmitia *et al.*, 1995). This is illustrated by our observation that non-physiological sprouting of processes are from the cell body which is not normally seen in differentiated neuronal cultures. The second possibility is that S100β is a modulator of tau phosphorylation and that any changes in their interaction could be a factor in the AD-related hyperphosphorylation as has been previously suggested (Baudier *et al.*, 1987a; Sorci *et al.*, 2000). Furthermore, the ability of S100β to inhibit PKC may potentiate the aberrant phosphorylation at key sites (*e.g.*, residues 262 and 313 (Correas *et al.*, 1992; Singh *et al.*, 1996b)). However, in our *in vitro* studies, S100β did not appear to promote aberrant phosphorylation, as indicated by the lack of AT8 staining which identifies PHF-tau related phosphorylated epitopes (Biernat *et al.*, 1992). Neuritic development, while beneficial in the short term to rejuvenate lost neuronal connections, can also be detrimental in the chronic stages of AD as it increases cellular metabolic requirements and exposes the neurons to external insults.

Initially, our finding that S100β failed to bind AD-derived tau was attributed to the

reduced number of neurons, which is associated with the progression of AD. This did not appear to be the case as the normal binding could be restored following alkaline phosphatase treatments. While this may suggest that all phosphate groups on tau hinder S100β binding, this is not evident as tau is naturally phosphorylated, and this does not affect binding of the control sample tau to S100β. In these studies, it is only with abnormal hyperphosphorylation of tau present in AD that prevents S100β-tau binding activity. Our study has also demonstrated that zinc is important factor in the internalization of S100β into neurons and enhances tau binding. In addition, we observed an increase in neuritic sprouting in SH-SY5Y cells treated with S100β and zinc, which suggests that metal binding may be critical to this outgrowth activity.

S100β has been demonstrated to have several biological functions in AD. This is reflected by its ability to bind zinc and calcium, as well as inhibit certain phosphorylation pathways. In addition, S100β has been shown to activate the complement pathway through interleukin–6 activation (Mrak *et al.*, 1995; Hays, 1998; Sheng *et al.*, 1996a; Sheng *et al.*, 1996b; Stanley *et al.*, 1994). S100β itself is activated by interleukin-1 and may also participate in a positive feedback loop, thereby inducing its own production through the promotion of astrocytic activity (Mrak *et al.*, 1995). In AD, the observed increase in S100β production appears to be related to some of the physiological changes associated with interleukins and to the increase in neuritic sprouting. The uptake of S100β may represent a key role in its ability to alter the neuronal activity. Our immunofluorescence data suggests that S100β uptake by cells is enhanced by the addition of zinc. As stated previously, zinc causes S100β to undergo a conformational change exposing a hydrophobic domain that

facilitates neuronal internalization. Within the cell, S100β may alter many cellular processes, including binding to tau.

The metal binding capacity of S100β appears to be a crucial functional element and may have some bearing on other disease pathways. S100β-calcium effects have been extensively examined by Baudier and Cole (1987a; 1987b; 1988), where they found evidence of S100β–calcium binding to microtubule associated proteins, including tau, and calcium/calmodulin-dependent protein kinase II. Calcium is also thought to be excitotoxic in AD (Kim *et al.*, 2000). In AD, both calcium and zinc have been implicated in the Aβ toxicity pathway. Zinc, as well as copper, is believed to accelerate the formation of Aβ fibrils (Bush *et al.*, 1994; Yang *et al.*, 2000). Aβ is implicated as a potential membrane protein that may promote the influx of calcium across the plasma membrane. The increase of S100β in AD may contribute to the shuttle of these metals to points of interaction thereby accelerating the pathogenic process.

Zinc does not normally appear in the cell as a free, or unbound, form. It is believed to be toxic in this state. This may be related to the ability of free zinc to enter via AMPA channels (Sensi *et al.*, 1997; Yin *et al.*, 1998; Sensi *et al.*, 1999), promoting excitotoxicity. Proteins such as metallothionein and S100β are induced by astrocytes in order to compensate for the extrusion of zinc into the extracellular space in order to block its toxic effects. In the case of S100β, the effect may detrimentally alter the disease process.

The role of zinc in AD has generated several interesting and pathogenically significant hypotheses. The potential role that it may play with S100β on the effect on neuritic sprouting is another important addition to this metal's possible role in the disease process. Finally, our observations suggest that, in addition to its activation of cytokines,

S100β may also play a more direct role in tau-related pathways that are associated with neurodegeneration in Alzheimer's disease.

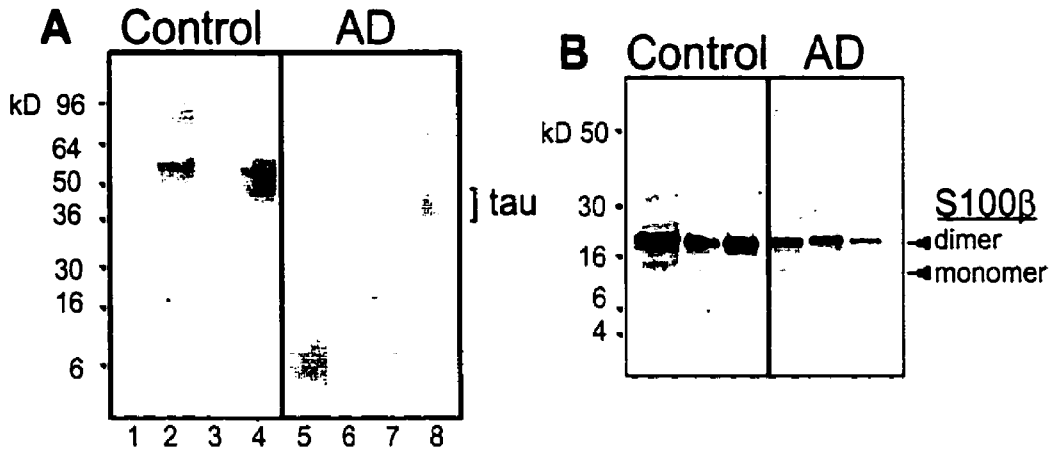

Figure 5.1: Affinity chromatography using immobilized S100β for identification of binding proteins **(A)**. Immunoblotting of zinc (**lanes 1,3,5 and 7**) and EDTA (**lanes 2,4,6 and 8**) eluted fractions indicated a significant amount of S100β-associated tau in control samples from both frontal (**lane 1,2**) and temporal cortices (**lane 3,4**). Comparable affinity analysis with AD-extracted proteins from frontal (**lane 5,6**) or temporal (**lane 7,8**) cortex indicated only weak tau immunoreactivity consistent with a reduced interaction with S100β. Zinc treated samples did not elute any proteins with tau immunoreactivity. Immunoblotting of total brain homogenates from AD and control indicating the elevated levels of S100β, as has been previously demonstrated by Griffin *et al.*, 1989 **(B)**.

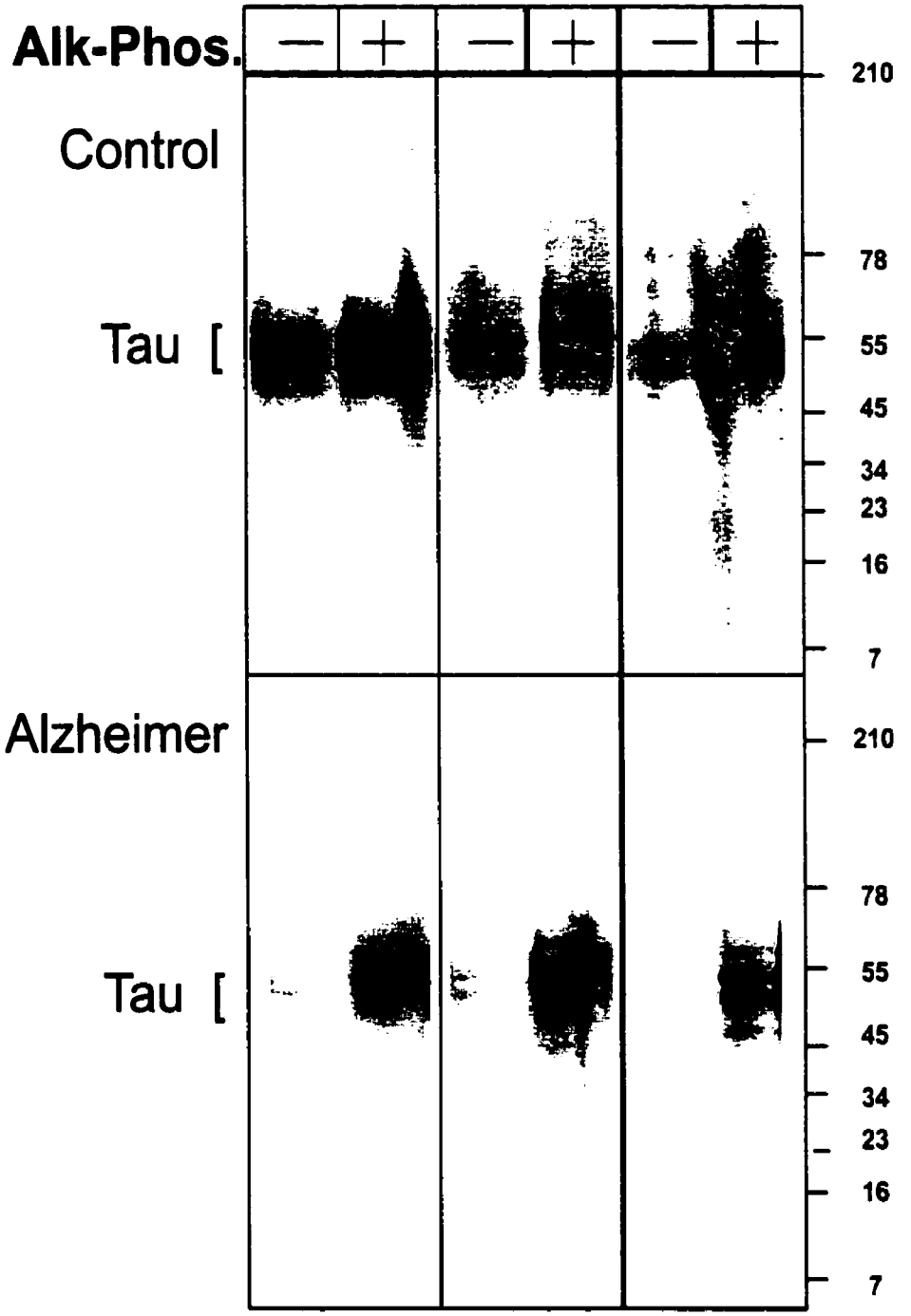
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Figure 5.2: Immunoprecipitation of S100β complexed with brain extracted tau from control and AD cases (3 separate tissue samples). Purified S100β incubated with tau-enriched and precipitated with an S100β polyclonal antibody indicated significant interacted evidenced by the co-precipitating tau. Untreated AD extracts displayed reduced tau binding to S100β under comparable conditions. The association was restored by dephosphorylation of the tau-containing extracts using alkaline phosphatase (Alk-Phos).

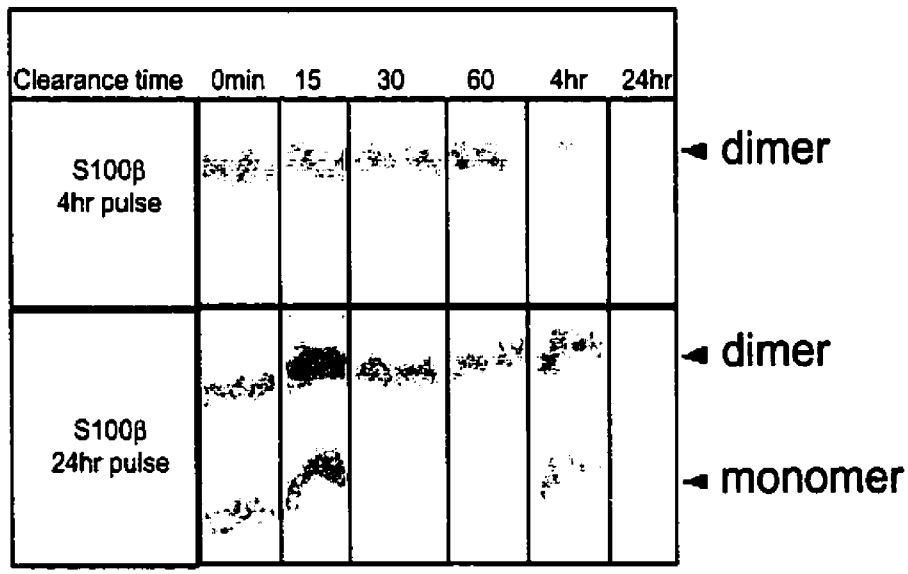

Figure 5.3: Time course of S100β internalization and clearance from differentiated SH-SY5Y neuroblastoma cells that were pre-incubated with S100β for 4 or 24 hrs. Lysates were examined at different time points (0, 15, 30, 60 minutes, 4 and 24 hrs) after the removal of S100β from the culture medium. Readily detectable S100β (monomeric/dimeric forms) were observed following the 24 hr pulse and to a lesser extent following 4 hrs pre-incubation.

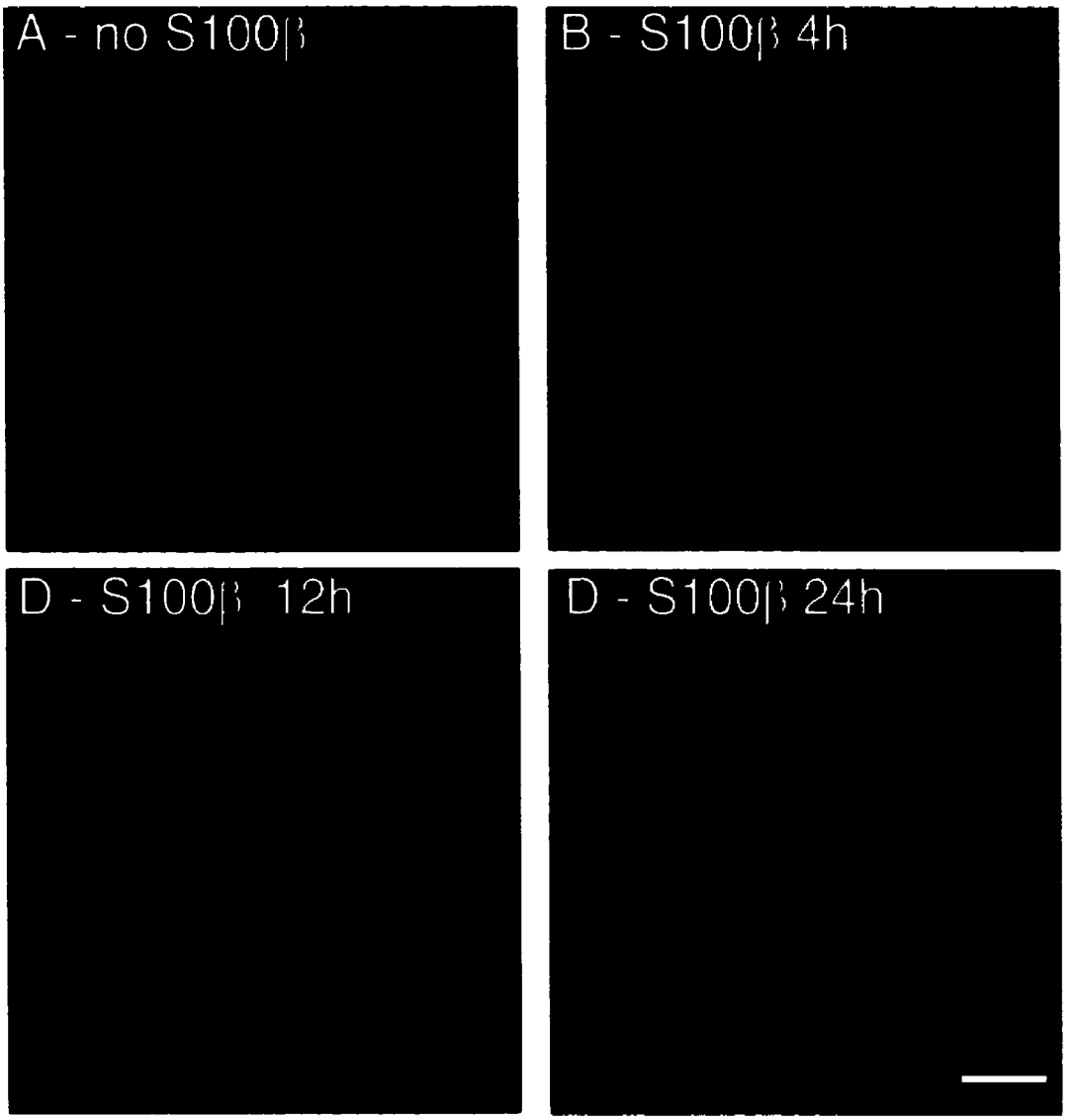

Figure 5.4: Immunofluorescence of SH-SY5Y cells which were pre-incubated with S100β for various lengths of time. Untreated cells displayed very low levels of S100β **(A),** which were increased following addition of S100β to the medium and incubations for 4 hrs **(B)** and 12 hrs **(C).** The S100β levels were significantly increased following 24 hrs of incubation **(D).** S100β was distributed within the cell body and processes consistent with the internalization of the protein rather than cell-surface association. Scale bar equals 10 m.

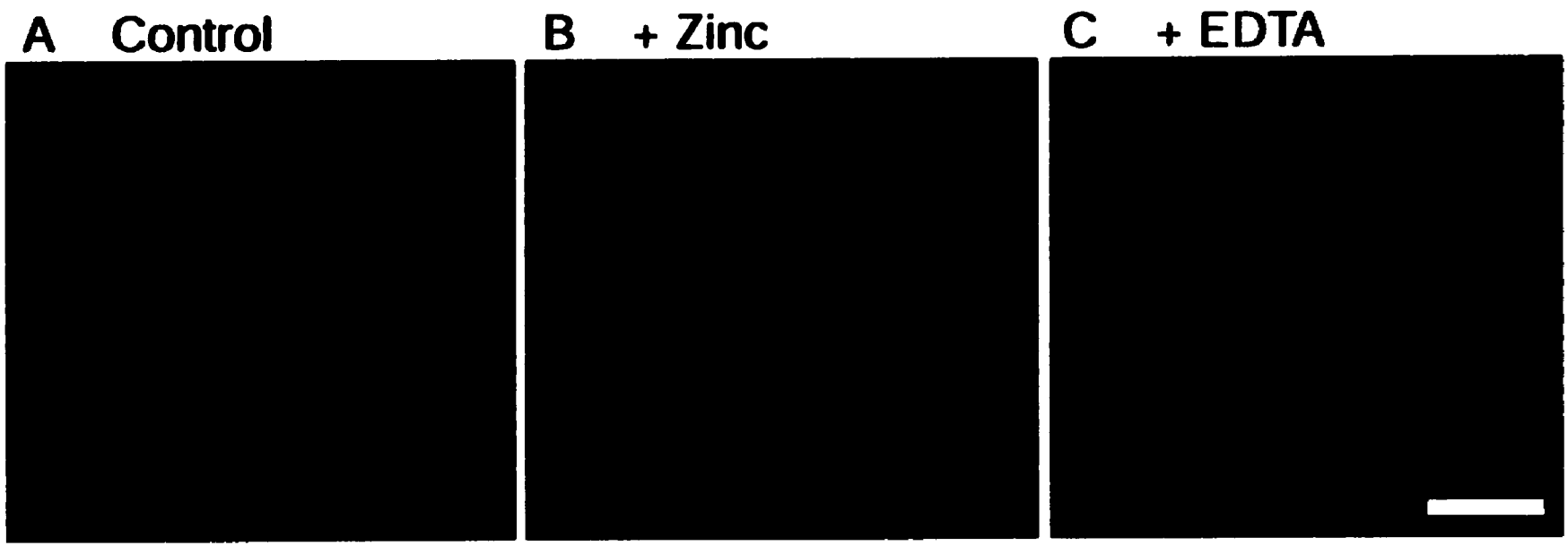
A Control
B + Zinc
C + EDTA

Figure 5.5: Immunofluorescence of differentiated SH-SY5Y cells demonstrating the effects of zinc on S100β internalization. Samples exposed to untreated S100β showed an easily detectable level of protein uptake at 24 hrs **(A)**. Elevation of the culture medium zinc concentration to 10μM resulted in a substantial increase in the intracellular S100β levels **(B)**. This zinc-induced enhancement of S100β internalization could be reversed with addition of metal chelators such as EDTA **(C)**. Scale bar equals 1 μm.

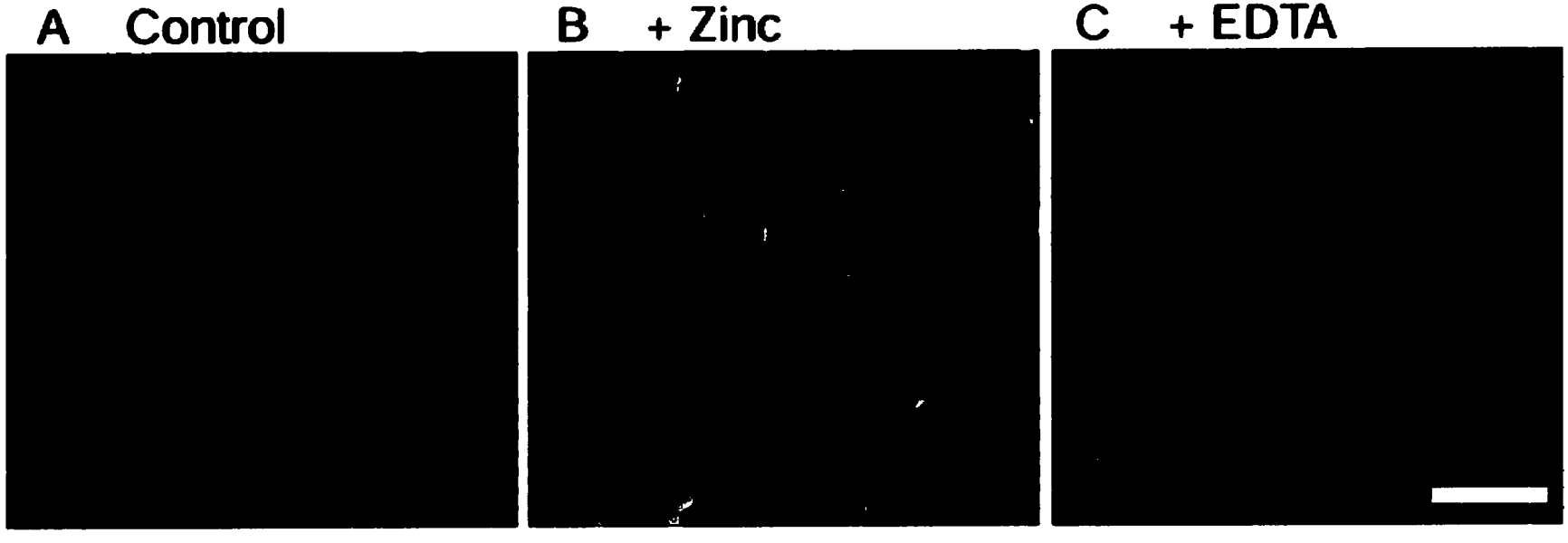
A Control
B + Zinc
C + EDTA

Figure 5.6: Co-localization of internalized S100β with tau in differentiated neuroblastoma cells. Under control conditions, S100β **(in red)** that was taken up by the cells showed partial overlap with tau **(green)** suggesting a possible intracellular association **(A)**. The co-localization was more pronounced with the addition of zinc to the culture medium **(B)**. Zinc elevated levels of S100β resulted in increased neurite outgrowth and frequent overlap of S100β with tau in these processes which appear as discrete, punctate staining within the cell processes **(B, arrows)**. Addition of EDTA to the culture medium prior to incubation of the cells with S100β, eliminated the tau co-localization pattern due to reduced protein uptake **(C)**. Scale bar equals 10 μm.

A - Control
B - with S100ß
C - S100ß + zinc

Figure 5.7: Stimulation of neurite outgrowth in SH-SY5Y cells following S100β internalization. Retinoic acid differentiated cells displayed a neuron-like morphology but with only a limited number of extensions **(A, arrow)**. With the addition of untreated S100β, the number and length of the processes were enhanced **(B)**. Addition of zinc to the medium and the accompanying increase in S100β uptake resulted in widespread increase in neurite outgrowth leading to the formation of dense networks of cell processes **(C)**. Cells and processes were visualized by immunofluorescence staining of the cell surface cadherins. Scale bar equals 10 μm.

Chapter 6

Authors - Haung Yu, Lynle Go, Barbara A Guinn, Paul E. Fraser, David Westaway and JoAnne McLaurin

Title - Phenotypic and functional changes in glial cells as a function of age.

Journal – Neurobiology of Aging (accepted with minor revisions, 2001)

Contribution to Paper –

1) Extracted and cultured astrocytes from Fischer 344 rats

2) Performed assays

3) Helped edit manuscript.

Significance of Article to Thesis –

This paper identifies a functional and phenotypic alteration in astrocytes associated with aging. Changes in astrocytes result in less responsive and protective capacities, thereby permitting neuronal death via Aβ toxicity. The work identifies the upregulation of interleukin 1β (IL-1β) in aging. IL-1β has also been shown to be increased in AD, upregulate S100β, as well as inhibit MT-III, expression. The increased levels of IL-1β may precede alterations in levels of metal binding proteins. This in turn may result in a cascade of biochemical events associated with AD as a result in MT-III reduction and S100β overproduction which have been examined in previous chapters. Furthermore, the alteration in astroglial populations, i.e. from mainly astrocytes in pups to microglia in aged animals may represent an important shift in neuroimmunological and protective function and may directly affect the progression of AD and other diseases associated with aging.

6.1 ABSTRACT

Both *in vivo* and *in vitro* investigations point to an important role for the immune system in the development of age-related neurodegeneration. Microglia isolated from aged female F344 rats, 18-20 months, show a higher percentage of cells with an amaeboid morphology indicative of activation, whereas, astrocytes had a quiescent morphology. The ability of astrocytes and microglia to attenuate toxin-induced neuronal injury was examined. Post-natal day 1-3 pup cells optimally rescued neurons from Aβ–induced toxicity, whereas mixed glial cells from 18-20 month old rats were unable to rescue PC-12 cells from Aβ-induced toxicity. Our results suggested the appearance of a neurotoxic co-factor, therefore we investigated the basal level of nitric oxide and pro-inflammatory cytokines to determine if altered levels of immune mediators play a role in the toxicity. Mitogen-stimulated nitric oxide production increased with age of donor, whereas, only the pup cells expressed an increase in TNF-α production. Basal levels of pro-inflammatory cytokines, as measured by RNA protection assays, increased with age and may contribute to enhanced susceptibility to neurodegenerative diseases.

6.2 INTRODUCTION

Alzheimer's disease (AD) is an important neurodegenerative disease, which is characterized by; a loss of neurons, neurofibrillary tangles and extracellular plaques both diffuse and senile (Mann, 1989; Masters *et al.*, 1985; Price *et al.*, 1991; Yamaguchi *et al.*, 1989). The major component of plaques is amyloid beta peptide (Aβ) a 40-42 residue peptide generated as a normal cleavage product of amyloid precursor protein (APP) metabolism (Esch *et al.*, 1990; Haas *et al.*, 1992). Theories of AD propose that Aβ plaque deposition or partially aggregated Aβ trigger a neurotoxic cascade thereby causing neurodegeneration. Evidence for this theory is based on *in vitro* studies demonstrating that Aβ is toxic to neurons and that increased release of Aβ, as a result of familial gene mutations, cause a more severe clinical course (Cai *et al.*, 1993; Citron et al, 1994; Citron *et al.*, 1997; Duff *et al.*, 1996; Lorenzo and Yankner, 1994; Pike *et al.*, 1993; Selkoe, 1996; Simmons *et al.*, 1994; Suzuki *et al.*, 1994). Direct injection of Aβ into rodent and primate brain has shown that plaque equivalent concentrations of Aβ resulted in profound neuronal loss and microglial proliferation of old primates, whereas, Aβ was non-toxic to young adult primates (Weldon *et al.*, 1998; Guela *et al.*, 1998). These results suggested that Aβ neurotoxicity *in vivo* is a pathological response of the aging brain, and thus longevity may contribute to the unique susceptibility and increased exposure to Aβ.

Investigations also point to an important role of the immune system in the development of AD symptoms and the rapidity of the disease's clinical course. Microglia and astrocytes are the immune mediators of the brain and are strategically located relative to all pathological structures in AD (DiPatre and Gelman, 1997; Mrak *et al.*, 1996; Sheng *et al.*,

1996; Sheng *et al.*, 1997. Senile plaques of AD patients contain deposits of immunological factors, such as, complement components, cytokines, growth factors and acute phase proteins (McGeer *et al.*, 1989; Sipe, 1997). *In vitro* studies have demonstrated the ability of Aβ to induce the release of pro-inflammatory cytokines, such as interleukin-1beta (IL-1β), tumour necrosis factor-α (TNF–α) and nitric oxide (NO) from early post-natal microglia and astrocytes (Akama *et al.*, 1998; Griffin *et al.*, 1997; Meda *et al.*, 1995). Correlation of the level of cytokines with the extent of cognitive decline has also been studied. Transgenic mice over-expressing IL-6 and TNF-α exhibit age-related deficits in conditioned avoidance, reduced long term potentiation and alterations of the exploratory and displacement behaviour (Campbell, 1997). Chronic inflammation within the brain of young rats produced an increase in the glial cell activation, induction of IL-1β, TNF-α, and APP mRNA, degeneration of hippocampal CA3 pyramidal cells, and a significant impairment in spatial memory (Hauss-Wegrzyniak *et al.*, 1998). These characteristics are all components of the neurobiology of AD and lend support for an inflammatory component leading to the associated cognitive deficits. Thus, evidence supports potential roles for over-expression of the inflammatory cytokines IL-1β, IL-6 and TNF-α in the pathogenesis of cognitive decline and AD.

Neurons rely upon glial cells for the maintenance of a healthy extracellular environment, for production of growth factors and removal of toxic substances (Jeohm *et al.*, 1998; Zietlow *et al.*, 1999). The interdependence of neurons and glia can be seen in the regulation of each other's functioning. The purpose of this study was to examine the ability of glial cells isolated from young, adult and aged female F344 rats to protect neuronal cultures from Aβ-induced toxicity and to examine the glial cell response to Aβ. Our data

indicate that age plays a role in Aβ-induced toxicity and that this may be linked to a higher basal expression of pro-inflammatory cytokines.

6.3 METHODS

6.31 Aβ Peptides

Aβ40 and Aβ42 were synthesized by solid phase Fmoc-chemistry by the Hospital for Sick Children's Biotechnology Centre (Toronto, ON). Peptides were purified by reverse phase HPLC on a C18μ bondapak column. Peptides were initially dissolved in 0.5 mL of 100% trifluoroacetic acid (24; TFA, Aldrich Chemicals, Milwaukee, WI), diluted in distilled H_2O, flash frozen and immediately lyophilized. Peptides were then dissolved in 40% trifluoroethanol (TFE, Aldrich Chemicals) in dH_2O and stored at -20 °C until use.

6.3.2 Gial Cell isolation

Primary young rat brain cultures were generated using a modification of the technique described by McCarthy and deVellis (1980). Newborn rat cerebral hemispheres, and brain stems were removed and placed in Ham's F12 buffered with 20 mM HEPES, containing penicillin/streptomycin. The tissues were gently forced through a 230 μm Nytex mesh with a glass rod, and subsequently passaged through 10 ml pipettes. The resulting cell suspension was filtered through 100 μm and 60 μm Nytex meshes, and centrifuged (1000 rpm for 7 min). The pellet was resuspended in complete DMEM and plated on poly-L-lysine coated 75 cm^2 flasks and maintained for 7 days in complete media. The culture media was changed on the 3rd day of culture and every 2 days thereafter. After confluence was reached the cultures were shaken at 225 rpm for 3 hr to remove loosely adherent cells which were discarded. The small bright or dark cells growing on top of the adherent astrocytes, consisting mainly of oligodendrocyte precursors and microglia, were subsequently removed by shaking at 260

rpm for 18 hr. After filtration through a 30 μm Nytex mesh to remove large cell aggregates, microglia cells were separated from oligodendrocyte precursors by plating on bacterial grade petri dishes for 3-5 hrs. Under these conditions microglia adhered to the plastic and oligodendrocyte precursors remained in suspension or were loosely adherent. The floating cells were removed and microglia were fed complete DMEM (Cohen and Alzaman, 1992).

Cultures of mixed (astrocyte/microglia) and enriched astrocyte or microglia cultures from F344 rats of adult and aged rats were established using a modification of the procedure for human glial cells (McLaurin *et al.*, 1995; Yong and Antel, 1992). Briefly, tissue was treated with 0.25% trypsin in the presence of DNAse (50 μg/ml), followed by a percoll gradient centrifugation at 15,000 rpm for 30 min. The resulting dissociated cell suspension comprised of mixed glial cells was then suspended in DMEM supplemented with 10% FCS, and seeded onto 25 cm^2 culture flasks. The following day the floating cells consisting of mostly oligodendrocytes are removed. Remaining cells consisting of microglia and astrocytes were allowed to develop morphologically for 7 days and isolated as for the young glial preparations.

6.3.3 Toxicity Assays

PC-12 cells were plated at 500 cells per well in a 96 well plate and suspended in 30 ng/ml NGF (Alamone Labs, Israel) diluted in N2/DMEM (Gibco BRL). Cells were differentiated over 5-7 days to a final cell count of 10,000-15,000 per well. Glial cells were added to fully differentiated PC-12 cells at 0-10,000 cells per well and left overnight to differentiate. Aβ was added to cultures at a final Aβ concentration of 0.1 μg/μl and

incubated for 24 hrs at 37 °C. Toxicity was assayed using the sulfhydrylrhodamine B (SRB) assay and the lactate dehydrogenase assay (LDH).

6.3.3.1 SRB Assay - Cells were fixed with TCA at a final concentration of 10%. Plates were washed with H_2O and air-dried. Protein was stained with 0.4% SRB (Molecular Probes Inc) in 1% acetic acid for 30 min (54). Plates were washed with 1% acetic acid and air-dried. The dye was extracted in unbuffered 10 mM Tris and absorbance was measured at 550 nm on a BIORAD Benchmark microtitre plate reader.

6.3.3.2 LDH Assay- Prior to addition of Aβ, FCS was added to cultures to a final concentration of 1% in order to stabilize LDH in the supernatant. Supernatants from the Aβ-treated cultures were removed and analyzed for LDH release using a commercial kit (Sigma). Results are expressed as B-B Units/ml.

6.3.4 Morphological Assay

Primary cultures of astrocytes were plated on poly-L-lysine coated coverslips at $5x10^4$ cells per coverslip. Cells were treated with Aβ peptides alone and in the presence of okadaic acid, okadaic acid alone or in media. Okadaic acid was used for inhibition of morphological staining at concentrations of 10-200 nM. After 4-24 hrs cells were fixed and stained with SRB as a general protein stain or with anti-GFAP antibody (Dako, Mississauga, ON) followed by secondary antibody linked to Cy3. Morphology was evaluated by light or fluorescence microscopy whereby total and stellate astrocytes were counted in 10 fields of at least 100 cells per coverslip.

6.3.5 Proliferation Assay

The proliferation rates of the isolated glial cells in the exponential growth phase were measured following the dilution of the culture to obtain $1x10^5$ cells in a final volume of 100 µl of culture media. Following an 18 hr incubation with 1µCi of [^{3}H] thymidine (Amersham, Oakville, Ontario, Canada) the supernatant was harvested and the amount of [^{3}H] bound to DNA was counted in a Topcount scintillation counter (Canberra-Packard, Mississauga, Ontario, Canada). Appropriate controls were used to detect background levels of [^{3}H] thymidine.

6.3.6 Oxidative Burst Assay

Nitroblue tetrazolium (NBT) acts as a nonspecific electron acceptor in biochemical pathways activated during the oxidative burst, producing a blue precipitate within the reactive cell (Badway *et al.*, 1986). NBT was dissolved in 0.9% NaCl at a final concentration of 1 mg/ml. PMA, a potent protein kinase C activator, was used at a final concentration of 10 nM or 100 nM. Mixed glial cultures, $2x10^4$ cells were seeded on coverslips, incubated with 30 µl NBT and either 100 µM phorbol myristic acid (PMA) or 10 µg/ml lipopolysaccharide (LPS). After 45 minutes, cells were fixed in 4% paraformaldehyde and mounted. Coverslips were examined using a light microscope and results expressed as the percent of cells with reduced NBT in their cytoplasm.

6.3.7 Cytokine Assays

6.3.7.1 TNF Assay

TNF was determined by its cytotoxic effect on the TNF sensitive mouse L929 cell line. Cells were incubated with serial dilutions of test supernatants and viability was measured after 24 hrs using the release of lactate dehydrogenase from dead or dying cells. LDH release into the media of cultures was evaluated using a commercial kit (Sigma) and results are expressed as B-B units.

6.3.7.2 Nitric Oxide (NO) Analysis

Glial cells were plated onto 96 well plates at 10^5 cells per well in 200 µl of serum free media and incubated alone, in the presence of PMA or LPS for 24 hrs. Supernatants were harvested and stored at 4°C until assayed. NO content was determined using the Greiss reagent against a standard curve generated with 0-50 µM sodium nitrite in media. 100µl of each sample was mixed with 50µl Greiss reagent and incubated for 5 min at room temp. Absorbance was read at 540 nm on a BIORAD Ultramark microtitre plate reader.

6.3.7.3 RNA Protection Assay

The level of cytokine RNA in glial cells isolated from young, adult and aged rats was measured using the Pharmigen Multi-probe RNAse protection assay using the RIBOQUANT rCK-1 template set. RNA isolated from the three glial populations was hybridized overnight with the ^{32}P-labelled probes, treated with RNAse to digest unprotected RNA, and electrophoresed on the Quick Point gel system (Novex, San Diego, CA) with pre-cast denaturing polyacrylamide gels. Autoradiography was performed on the gel for up to 48 hrs.

6.4 RESULTS

6.4.1 Aβ-induced Toxicity and Morphological Changes as a Function of Aging

Glial cells were isolated from day 1-3 pups, 3 month old adult and 18 month old F344 rat brains and allowed to differentiate in culture for 7 days. The number of microglial cells present in the aged mixed glial preparations was higher than in the adult and pup cultures, therefore in all mixed glial cultures we controlled for this ratio by further purification of pup and adult cultures. This is achieved by differential adherence properties after 7 days of culture, the less adherent microglial cells along with non-differentiated astrocytes can be removed by rotational shaking overnight (McLaurin *et al.*, 1995; Yong and Antel ,1992). The resultant cultures contain a mixture of astrocytes and microglial that more closely resemble the aged mixed glial cultures.

In order to evaluate the role of glial cell aging in Aβ-induced neuronal toxicity, we co-cultured various aged glial cells with nerve growth factor (NGF)-differentiated PC-12 cells. We chose the PC-12 cell line as our neuronal target cell, since they have been extensively used in Aβ-toxicity studies (McLaurin *et al.*, 2000; Behl, 1997) and we could easily control the contribution of glial cells according to the number added. We recognize the limitations of an immortalized cell line, but the inherent limitations of using primary cultures due to differential susceptibility of various aged primary neurons (Brewer, 1998) and the presence of parental glial cells would complicate the interpretation of the role of externally added glial cells in our paradigm. Therefore, under basal conditions we evaluated the survival properties of co-culturing differentiated PC-12 cells with glial cells of various ages. No difference in survival of NGF-differentiated PC-12 cells in the presence of various

aged glial cells was detected over 72 hours using morphological evaluation (Fig. 6.1A-C), trypan blue exclusion and LDH assay as a measure of membrane damage and cell death (data not shown). In addition, PC-12 cells did not de-differentiate and proliferate in the presence of glial cells as determined using ^{3}H-thymidine incorporation assay (Fig 6.2). Of note is that the proliferation of pup derived glial cells decreased in the presence of NGF-differentiated PC-12 cells, suggesting that the glial cells are more quiescent. This phenomena has been reported previously for astrocytes co-cultured in the presence of primary neurons and thought to be attributed to neuronal-glial cross-talk (Jeohm *et al.*, 1998; Yong and Antel, 1992).

The ability of astrocytes and microglia to attenuate toxin-induced neuronal injury was measured as a function of neuritic dystrophy and cell swelling (Fig. 6.1) or membrane damage 24 hrs after addition of Aβ (Fig. 6.3). Since 5000 fetal cells optimally rescued PC-12 cells from Aβ–induced toxicity (Fig. 6.3A), we then examined the ability of 5000 mixed astrocyte and microglial cells isolated from aged animals to attenuate Aβ toxicity. The mixed glial cells from 18-month old rats were unable to rescue PC-12 cells from Aβ-induced toxicity and the extent of membrane damage was not altered (Fig. 6.3B). Mixed glial cultures isolated from adult F344 rat brain, were less able to protect neurons from Aβ-induced toxicity in comparison to pup cells but more protective than aged cells (data not shown). Bee venom mellitin was used as a control for killing, as this protein induces cell death via formation of plasma membrane pores (Fletcher and Jiang, 1993). These results suggest that the age-dependent mechanism may involve either a loss of a neuroprotective agent or the appearance of a neurotoxic co-factor associated with the glial cells.

In order to further characterize the response of various aged glial cells to Aβ, we evaluated the morphological transition associated with Aβ treatment of astrocytes. It has

been reported that treatment of postnatal astrocyte cultures with Aβ results in a morphological transition from a flat to a stellate morphology and is an indication of cell activation (Hu *et al.*, 1998; Pike *et al.*, 1994; Salinero *et al.*, 1997a). After treatment of enriched astrocyte cultures from pup, adult and aged brain with Aβ40 and Aβ42 for 24 hrs, we fixed the cells followed by GFAP fluorescence or sulfhydrylrhodamine B staining. Light microscopy demonstrated that approximately 25% of the pup cells underwent the morphological transition, whereas only 8% of the adult astrocytes underwent a similar change (Table 6.1). Aβ-induced morphological transition in pup and adult astrocytes could be inhibited by co-treatment with okadaic acid as had been previously reported (Salinero *et al.*, 1997b). In contrast, none of the aged astrocytes underwent a morphological transition even in the presence of a 10 fold greater concentration of Aβ42 (Table 6.1). Furthermore, the aged astrocyte cultures were incubated for an extended period of time up, to 72 hours, in the presence of Aβ in order to rule out the possibility that the time course for morphological change was altered. The lack of a morphological transition further suggests that the functioning of glial cells isolated from aged brain is different than that of pup and adult brain.

6.4.2 Functional Changes in Glial Cells during Aging

Light microscopic examination of the cells isolated from increasing aged rats demonstrated various morphologies of both astrocytes and microglia. In order to characterize a link between cellular morphology and activation, oxidative burst was used as a measure of basal activation and an early indication of activation after treatment with phorbol myristic acid (PMA) and lipopolysaccharide (LPS). The reduction of NBT after 30 minutes can be detected in individual cells by the presence of an insoluble precipitate. The pup cells

had a low basal burst activity which was distributed between astrocytes and microglia, whereas, the 3- and 18-month old glia had a higher basal level, 70.8% and 63.1% respectively, which was predominantly limited to microglia (Fig. 6.4). PMA stimulation resulted in an almost complete activation of pup glial cells, 93.1%, but only a small increase in the aged cells (Table 6.2). These results further emphasize the differences in activation of glial cells during aging and suggest altered responses to stimulation during aging.

We further examined the age-related changes in functional properties of mixed glial cultures and their response to mitogenic stimulation by LPS and PMA. Mitogen stimulation has been shown to up-regulate NO and TNF-α in both astrocytes and microglia (Murphy, 2000). In our cultures, mitogen-stimulated NO production increased with age of donor (Fig. 6.5A). Both adult and aged glial cells had a 6- and 10-fold increase in nitric oxide production over 48 hrs PMA stimulation, whereas the LPS stimulation was 5- and 4-fold, respectively. PMA has previously been shown to be a more avid promoter of NO synthesis than LPS, which correlates well with our result (Vincent *et al.*, 1998). Although pup glial cells had a low NO synthesis in comparison with adult and aged, these values are similar to those previously reported for post-natal astrocyte cultures (Paakkari and Lindsberg, 1995). The production of TNF-α by glial cells is measured as a function of cell death of the TNF-sensitive L929 cell line (Fig. 6.5B). Cell death in this case is accompanied by the release of LDH into the media, whereupon it is assayed against a TNF-α concentration curve. Pup glia do not express TNF-α basally but upon stimulation with PMA and LPS up-regulate TNF-α 2.5- and 3-fold above basal levels. Adult and aged glial cells expressed a high basal level of TNF-α which was unaffected by stimulation. Furthermore in contrast to pup cultures, aged glial expression of TNF-α was not down-regulated in the presence of α-lipoate or PDTC,

potent inhibitors of inflammatory reactions (Packer *et al.*, 1997; Kushura *et al.*, 1999; Fig. 6.5C). These results suggest that production and stimulation of cytokines may be altered during aging.

In order to further investigate basal cytokine synthesis with aging, we evaluated the RNA expression of the pro-inflammatory cytokines using a multi-probe RNA protection assay. The multi-probe kit includes IL-1α, IL-1β, TNF-β, IL-3, IL-4, IL-5, IL-6, IL-10, TNF-α, IL-2, IFN-γ, and the house keeping genes L32 and GAPDH (Pharmingen, SanDiego, CA). As demonstrated in a representative experiment in Figure 6.6, very low levels of cytokine mRNA species were detected in pup glial cultures, whereas the expression of the housekeeping genes L32 and GAPDH were strong. In contrast, mRNA species for IL-1α, IL-1β, IL-5, IL-6 and TNF-α were detected for both adult and aged glial cultures, and IL-3 was only expressed by aged cultures. Densitometry of several RNA protection assays demonstrate that the mRNA expression of all cytokines increased with age, IL-1α, IL-1β and IL-6 increased approximately 10-fold between pup and aged samples (Table 6.3). As demonstrated using the biological assay of TNF, the basal mRNA expression in both the adult and aged cultures was comparable. These results confirm the activation of glial cells as a function of age, which manifests as an increased basal expression of inflammatory mediators.

6.4 DISCUSSION

Our studies demonstrate one factor that contributes to the enhanced susceptibility of the aged brain to neurodegeneration is an altered immune function of glial cells. Glia isolated from aged F344 rats were unable to protect a neuronal cell line from Aβ-induced toxicity under conditions in which pup glia were proficient. In examination of glia isolated from aged brain, microglial cells had an activated morphology and represented a greater proportion of viable cells, this correlates well with *in vivo* studies, which have demonstrated an increase in the number of amoeboid microglia with age (DiPatre and Gelman, 1997; Rozovsky *et al.*, 1998). Studies in aged mice and rats have demonstrated that age-related activation of microglial cells is maintained in culture, as illustrated by the elevated IL-6 expression and resistance of glia to down-regulation by TGF-β (Blasko *et al.*, 1997; Salinero *et al.*, 1997a). In addition, astrocytes in our cultures had a quiescent morphology indicative of senescence. Quantitation of astrocyte numbers in aged CNS demonstrated that the number of S100β reactive cells does not increase with age, suggestive of a senescent environment (Sheng *et al.*, 1996; Tiu *et al.*, 2000). Microglial activation and astrocyte senescence in the CNS is also observed after sciatic nerve injury in aged rats (Gilmore and Knae, 1998). In contrast a previous report demonstrated that astrocyte proliferation was not inhibited by TGF-β and was suggested to be the result of astrocytic activation (Rozovsky *et al.*, 1998).

Age related changes have been extensively characterized in the periphery, where it has been demonstrated that an increase in the basal expression of inflammatory cytokines is associated with aging (Selkoe *et al.*, 1996; Rink *et al.*, 1998; O'Mahony *et al.*, 1998; Caruso *et al.*, 1996). This has been linked to a decrease in the control of IL-6 expression, probably

as a result of hormonal changes associated with menopause or andropause. IL-6 is a potent mediator of inflammatory processes and has been linked with several chronic diseases associated with aging, including Alzheimer's disease (Erscheler and Keller, 2000). Our results demonstrate that with age the expression of inflammatory mediators in the CNS, nitric oxide, IL-1α/β, IL-6, and TNF-α are increased and that the regulation of expression is different from young. Previous studies have demonstrated that IL-6 expression is increased in the brain of aged mice and can be attributed to microglial expression (Ye and Johnson, 1999). Under conditions that both up- and down-regulate cytokine expression in cultures isolated from young animals, cultures isolated from aged rats were unresponsive. The lack of stimulation at low μM activator concentrations, may be due to prior priming of the cells by the higher basal cytokine level present in the CNS of aged animals. This phenomenon has been demonstrated in macrophage cultures, in which priming of macrophages with IL-1β inhibited stimulation of serum amyloid A by IL-6 (Uhlar and Whitehead, 1999). Further characterization demonstrated that initial priming of immune mediators with one cytokine altered the response to alternate cytokines, mitogens or pathogens. This may help to explain the apparent conundrum in which IL-1β has been shown to mediate Aβ-induced morphological changes in young astrocytes whereas it had no effect on the aged astrocytes. Constant basal expression of cytokines may create a level of tolerance such that an increase in either cytokine expression or a related morphological change can only occur at much greater levels of stimulation. This hypothesis is supported by studies in the periphery that examined the activation of T-cells isolated from young adult, middle age and seniors in which stimulation of T-cells from elderly patients was seen only at 1000 fold higher concentrations than was active in young adults (Doria and Frasca, 1994). This alteration in

immune function has been suggested to contribute to the enhanced susceptibility of the elderly to bacterial, viral infections and neoplasias.

We would like to propose that the altered basal expression of cytokines in the CNS may contribute to the susceptibility of elderly patients to neurodegenerative diseases, such as Alzheimer's disease and reduction of protective capacity to toxic agents, such as Aβ toxicity. It has been shown that TNF-α augments Aβ induced apoptosis of the human neuroblastoma cell lines, SK-N-SH and DU-145 (Blasko *et al.*, 1997). Therefore, if the basal level of TNF-α is increased in the aging brain then introduction of increased concentrations of Aβ may increase the susceptibility of primary neurons to Aβ-induced apoptosis. The alterations that occur due to normal aging must be understood in order to determine their effect on disease processes, which occur during aging.

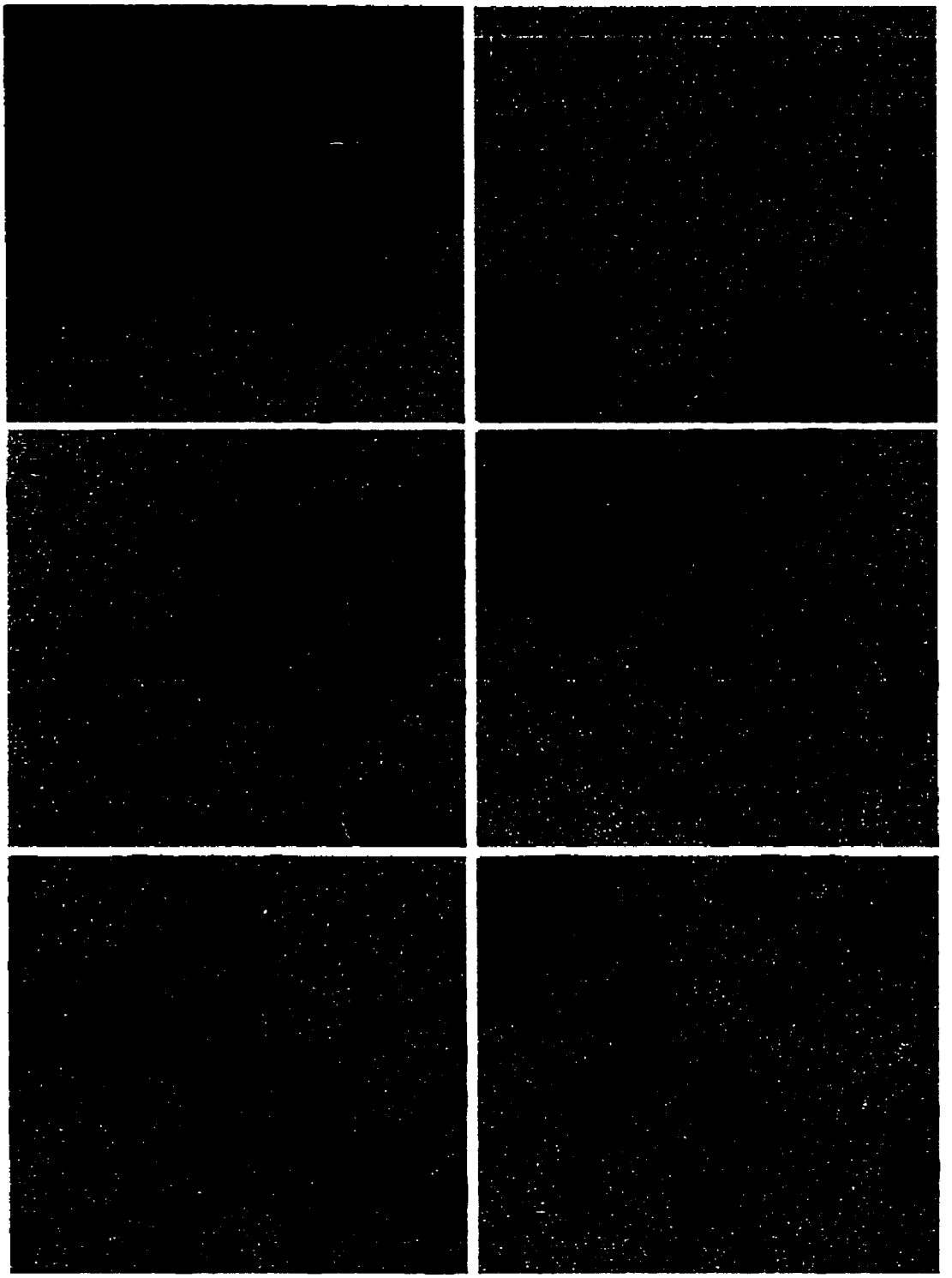

Figure 6.1: The survival of NGF-differentiated PC-12 cells alone (A) and in the presence of pup (C) or aged (E) mixed glial cultures was evaluated morphologically. No difference could be detected in the PC-12 morphology when co-cultured for up to 72 hrs with glial cells of various ages, as determined by sulfhydrylrhodamine B staining and examination by light microscopy. Toxin-induced (Aβ) injury was partially attenuated by the presence of pup glia (D) as compared to PC-12 cells alone (B) and aged glia (F). Magnification 40X.

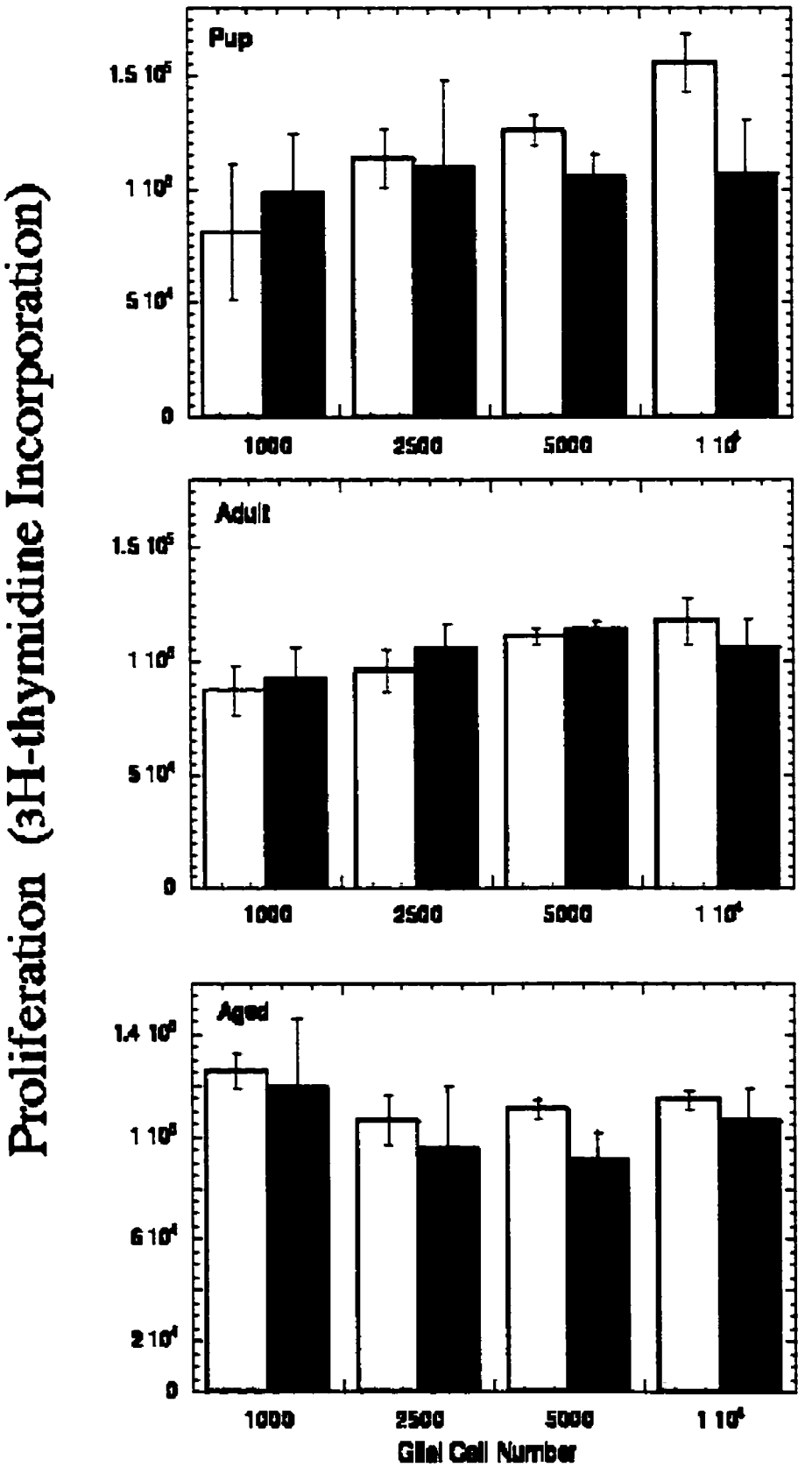
Proliferation (3H-thymidine Incorporation)
Pup
Adult
Aged
1000
2500
5000
Glial Cell Number

Figure 6.2: The proliferative rate of enriched glial cultures in the presence and absence of PC-12 cells was determined in an 18hr ^{3}H-thymidine incorporation assay. Pup glial cells alone (open bar) rate of proliferation increased with increasing cell number, whereas in the presence of PC-12 cells (solid bar) the rate of proliferation was constant. No difference could be detected in the proliferative rate of adult mixed glial cells in the absence or presence of PC-12 cells. The rate of proliferation of mixed cultures decreased with age and upon incubation in the presence of PC-12 cells. Data are expressed as the mean±SD.

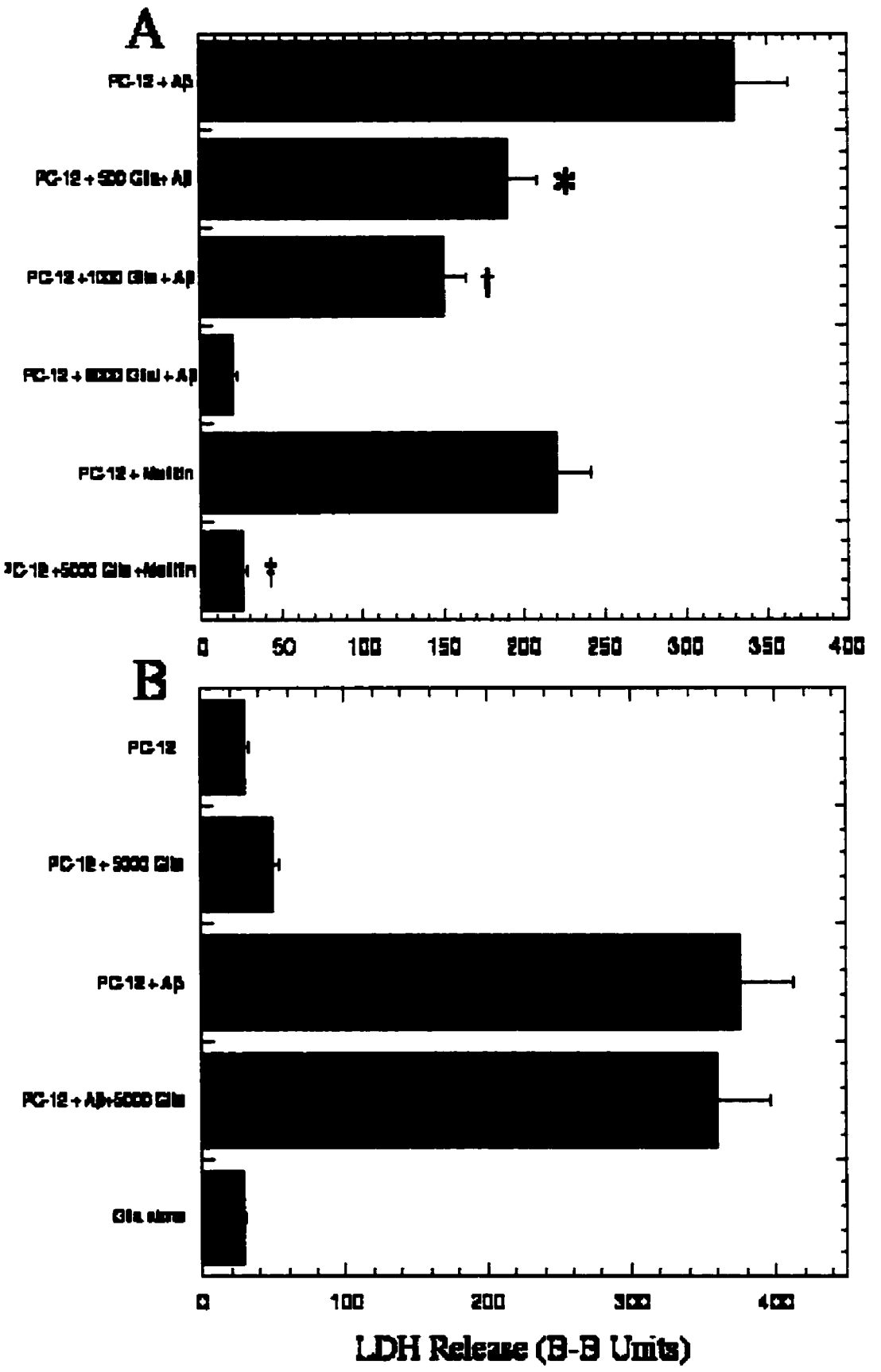
A
PC-12 + Aβ
PC-12 + 500 Glu + Aβ
PC-12 + 1000 Glu + Aβ
PC-12 + 5000 Glu + Aβ
PC-12 + Melittin
PC-12 +5000 Glu +Melittin
0
50
100
150
200
250
300
350
400
B
PC-12
PC-12 + 5000 Glu
PC-12 + Aβ
PC-12 + Aβ+5000 Glu
Glu alone
0
100
200
300
400
LDH Release (B-B Units)

Figure 6.3: The ability of mixed glial cells to attenuate Aβ-induced cell death was evaluated using the lactate dehydrogenase assay. Increasing the number of post natal day 1 glial cells attenuated both Aβ- and mellitin-induced toxicity as demonstrated by the decreased level of LDH released into the media (A). Aged mixed glia were unable to rescue PC-12 cells from Aβ-induced toxicity (B).Enzyme activity is expressed in B-B Units as per kit instructions. Data are expressed as mean±SD of at least 3 experiments. Paired student t=test *$p<0.01$, † $p<0.001$.

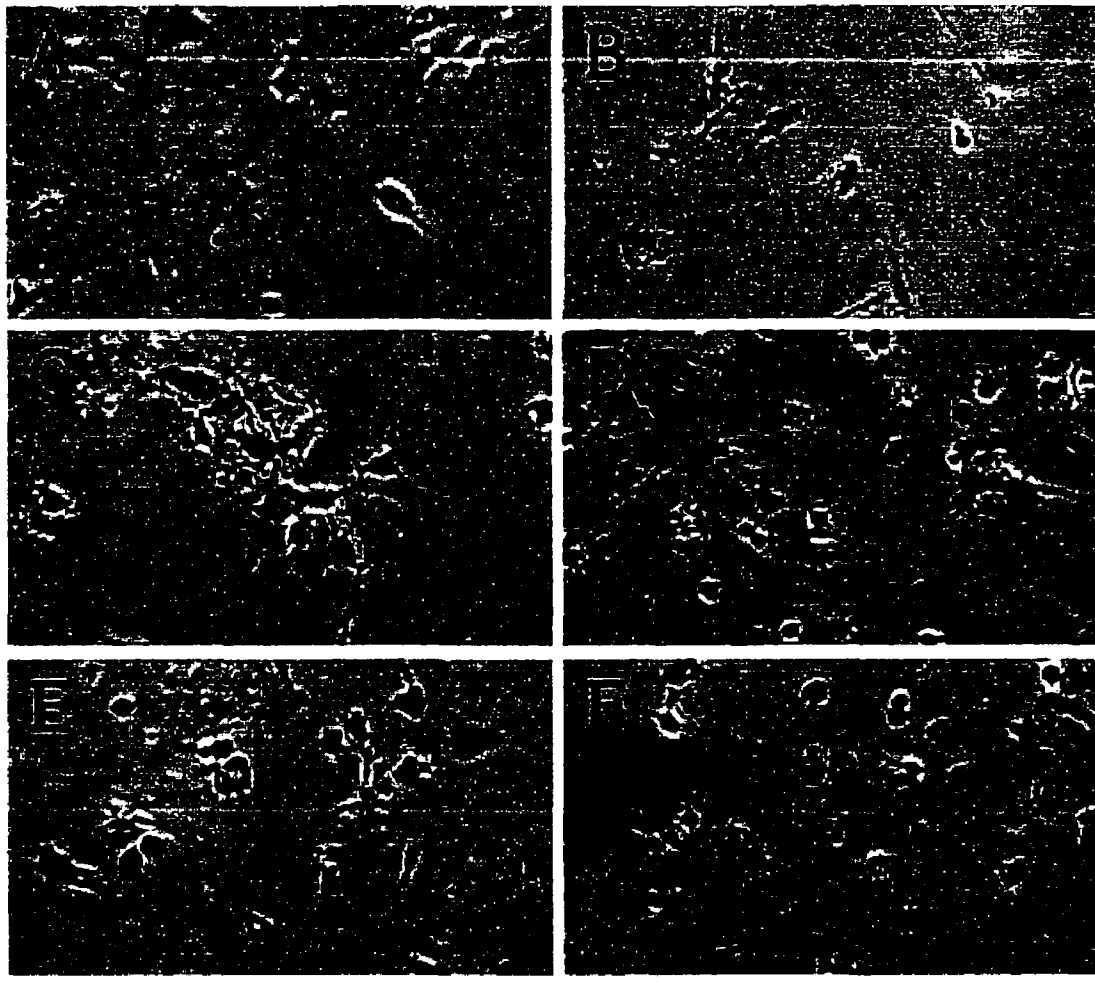

Figure 6.4: The extent of oxidative burst activity in mixed glial cultures, as measured by tetrazolium blue precipitation, was used as an indicator of basal and short-term activation. The ability of glial cells isolated from pup (A,B), adult (C,D) and aged (E,F) rats to undergo an oxidative burst was examined basally (A,C,E) and after PMA stimulation (B,D,F). The basal level of activation of these cultures increases with age as demonstrated by an increase in the number of cells containing the tetrazolium precipitate. It is also apparent that an increasing number of microglia with an activated morphology, ameboid, are present with increasing age.

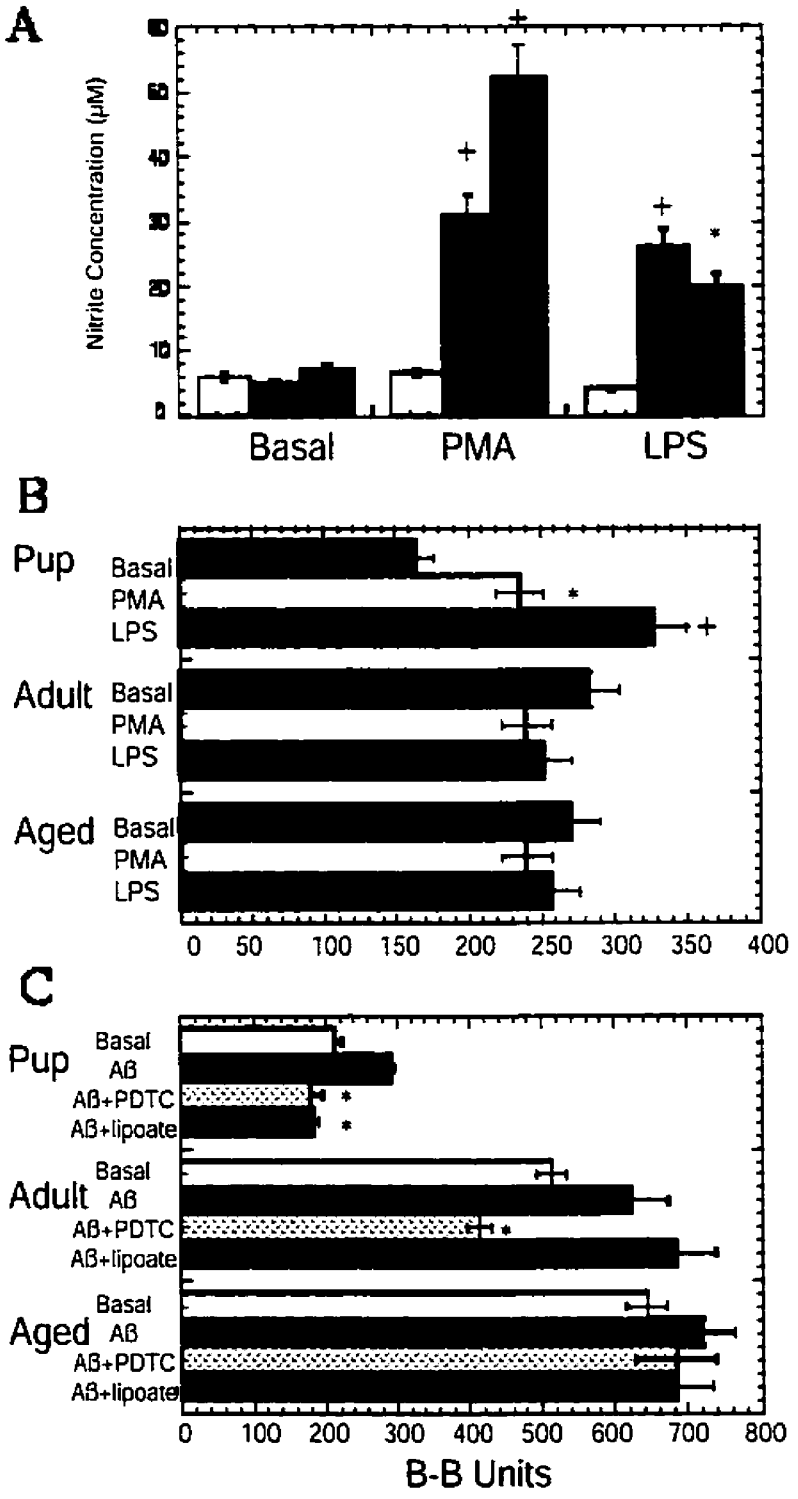
A
Nitrite Concentration (μM)
Basal
PMA
LPS
B
Pup
Basal
PMA
LPS
Adult
Basal
PMA
LPS
Aged
Basal
PMA
LPS
0 50 100 150 200 250 300 350 400
C
Pup
Basal
Aß
Aß+PDTC
Aß+lipoate
Adult
Basal
Aß
Aß+PDTC
Aß+lipoate
Aged
Basal
Aß
Aß+PDTC
Aß+lipoate
0 100 200 300 400 500 600 700 800
B-B Units

Figure 6.5: Glial cells isolated from rats of increasing age were examined for their basal and activated expression of nitric oxide and TNF-α. A) The mitogens, phorbol myristic acid (PMA) and lipopolysaccharide (LPS) were added to cultures for 48 hrs before evaluation of NO expression using the Greiss reagent. Both the basal and activated level of NO increases with age. Pup (open bars), adult (grey bars) and aged (black bars). B) TNF-α production by mixed glial cultures was measured using the survival of the TNF sensitive cell line, L929. Pup glial cells have a lower basal expression of TNF that is enhanced upon activation with PMA and LPS. Adult and aged glial cells have a higher basal level of TNF that could not be up-regulated by PMA or LPS in a 24 hr assay. C) TNF can also be stimulated by addition of Aβ. α-Lipoate and pyrrolidine dithiocarbonate (PDTC) have been shown to down-regulate cytokine production. These compounds were used to evaluate the cytokine expression in the presence of glial cells isolated from increasing aged rats. Down-regulation of TNF production could be achieved with pup cells but not aged cells. Adult cultures were downregulated in the presence of PDTC but not a-lipoate. Data for all experiments are the mean±SD, paired student t-test *$p<0.01$, † $p< 0.001$.

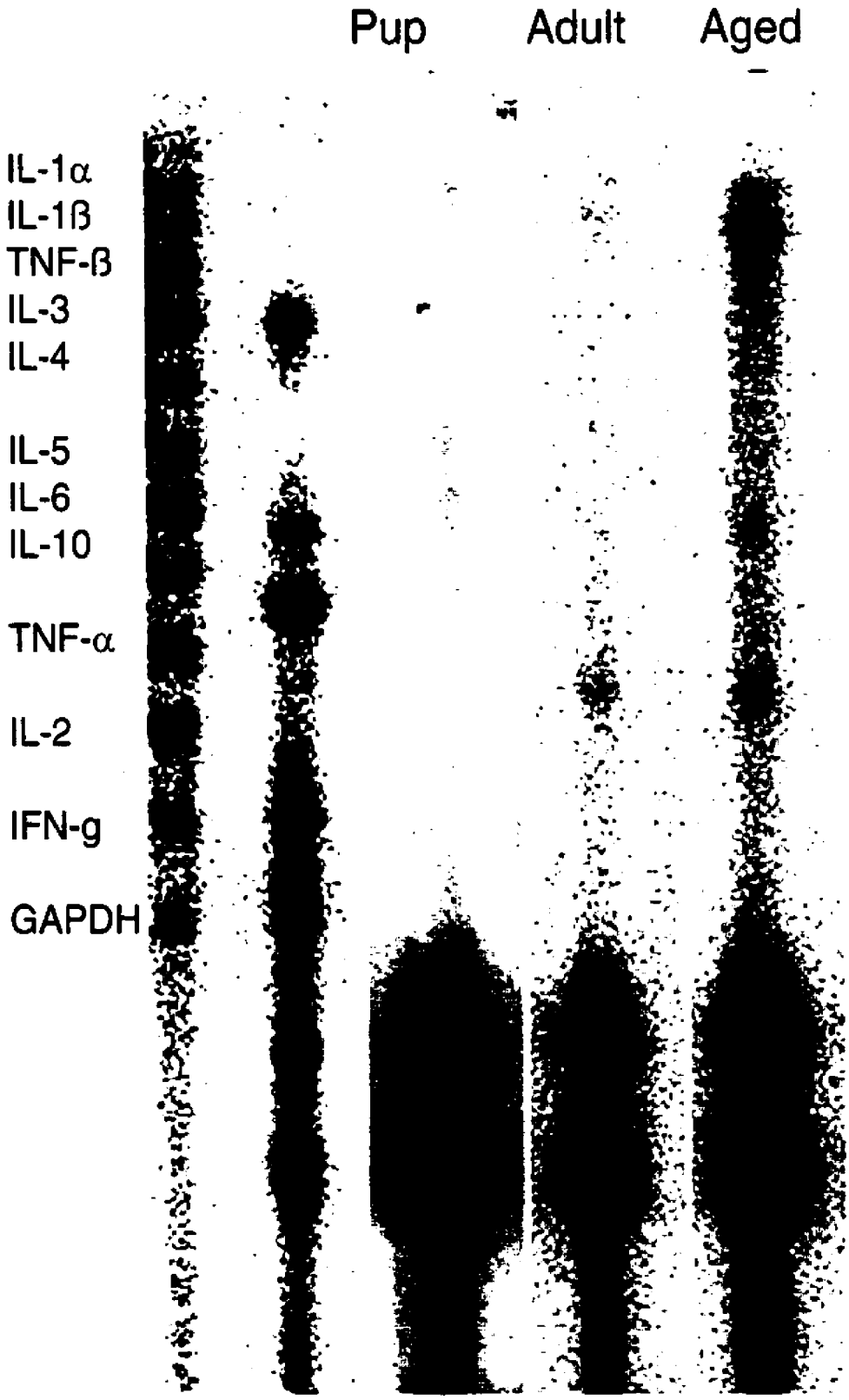
Pup
Adult
Aged
IL-1α
IL-1β
TNF-β
IL-3
IL-4
IL-5
IL-6
IL-10
TNF-α
IL-2
IFN-g
GAPDH

Figure 6.6: Representative autoradiograph of a multi-probe ribonuclease protection assay to screen for the expression of various cytokine mRNAs as a function of age. Protected mRNAs were visualized by autoradiography and quantitated using NIH image system, V1.59. Rat control RNA supplied by manufacturer and used as a positive control (lane 2), pup (lane 3), adult (lane 4) and aged (lane 5) glial cell cultures. It is of note that each probe band (lane 1) migrates slower than its protected band due to flanking sequences that are not protected by mRNA.

Condition	Morphological Change (% total cells)
Postnatal Day 1 Astrocytes	
Basal	6.6 +/- 2.3%
10 μM Aβ 1-42	25.8 +/- 6.2%†
Aβ1-42 + 10 nM Okadaic Acid	22.0 +/- 2.7%†
Adult Astrocytes (3 months of age)	
Basal	2.1 +/- 1.1%
10 μM Aβ1-42	8.39 +/- 4.5 %*
Aβ1-42 + 10 nM Okadaic Acid	1.54 +/- 2.0 %
Aged Astrocytes (20 month of age)	
Basal	2.0 +/- 1.9 %
10 μM Aβ1-42	1.3 +/- 1.1 %
Aβ1-42 + 10 nM Okadaic Acid	2.2 +/- 2.5 %

Table 6.1. Aβ1-42 induction of a morphological change in astrocytes is dependent on the age of the rat donor. F344 rat astrocyte cultures were treated for 24 hrs with 10 μM Aβ1-42 in the presence and absence of okadaic acid. Cells were fixed, and stained with GFAP before counting cells to determine the percentage of cells undergoing a morphological transition. Data is the mean +/- SD of 3 separate rat cultures. Paired student t-test against basal levels, *$p<0.01$, †$p<0.001$.

Condition	Oxidative Burst (% total cells)		
	Basal	LPS (10 μg/ml)	PMA (100 nM)
Postnatal Day 1			
Astrocytes	15.1±7.9	20.5±4.0	24.5±3.8
Microglia	19.0±6.5	22.6±3.7	85.9±4.3†
Adult			
Astrocytes	11.6±3.3	37.1±5.9†	22.4±5.3*
Microglia	17.1±3.5	28.5±1.8*	43.6±3.5†
Aged			
Astrocytes	66.3±2.4	67.1±8.1	88.3±3.7
Microglia	70.8±3.3	71.6±4.2	81.3±4.3

Table 6.2. Oxidative burst activity of glial cells isolated from various aged rat donors. Nitroblue tetrazolium reduction during the oxidative burst produces a precipitate within the reactive cell. Cells were incubated for 30 min at 37°C with NBT. At the end of the assay, the cells were fixed and examined by light microscopy. The results are presented as the percentage of cells with reduced NBT in their cytoplasm. Results represent the mean ± SD of at least three experiments. Paired student t test against basal levels, * $p<0.01$, † $p<0.001$.

Cytokine	mRNA Expression (% GAPDH)		
	Pup	Adult	Aged
IL1-α	1.5±1.0	6.7±1.6*	12.9±0.6†
IL-1β	3.9±0.8	13.0±7.1*	31.9±1.6†
IL-3	ND	ND	16.4±3.6
IL-5	ND	5.9±0.9	13.0±2.5
IL-6	2.6±0.9	6.6±0.6*	13.9±1.4†
TNF-α	0.5±0.2	17.0±1.7†	13.0±2.0†

Table 6.3. Cytokine mRNA expression in mixed glial cultures from F344 rats of various ages. Autoradiographs were scanned and analyzed using NIH image analysis software, all values were normalized to the GAPDH level to account for any subtle variations in RNA loading. Values represent the mean±SD of three readings. ND = not detected. Unpaired student t-test, * indicated where $p<0.01$ for adult versus pup, † indicated where $p<0.001$ for aged versus pup.

SUMMARY

7.1 Experimental Evidence of Neuronal Regulation by MT-III and S100β

These experiments provide insight into the roles MT-III and S100β have on neuronal activity. In order to establish if a deficit in MT-III expression exists, an extensive examination was performed. In Chapter 3, we identified that there is a reduction in DNA mononucleosomes, mRNA and protein MT-III in Alzheimer's disease. This study went beyond others as it incorporated every aspect of expression and employed more stringent conditions (such as post-mortem time and increased sample size). Furthermore, in Chapter 2, the antibody made to MT-III is characterized and shown to have a higher level of sensitivity based on a modified protocol used to examine hydrophillic small molecular weight proteins. As MT-III reduction in AD has been a contentious point, this study provided solid evidence of a downregulation of the protein. MT-III levels may be reduced in AD as a consequence of IL-1ß overexpression (Uchida, 1999). Additional work in this thesis, as well as others have demonstrated that there is an increase in IL-1ß levels in AD brain tissue (Sheng *et al.*, 1996b, Griffin *et al.*, 1994).

Post-mortem analysis, however, can be complicated by other variables such as cause of death, length of illness and drugs used chronically or prior to death. Appendix I provides information on these parameters, where possible. While the cause of death from patients with Alzheimer's disease is predominantly bronchopneumonia, this is not uniform. Control group brains came from individuals who died of cancer and heart attack, some following prolonged periods of arteriosclerosis. Duration of ailments can also be highly variable. Chronic ailments may reduce metabolic function and may also decrease RNA and protein production and degradation. The individuals in the AD group are more likely to suffer from

long-term ailments (AD as well as bronchopneumonia) prior to death, while the control group would not suffer as long. If the control group contains individuals that were more likely to die of sudden events, like heart attack without atherosclerosis or suicide, this may cause MT-III levels to be reduced in AD.

In addition to the neuroinhibitory function that MT-III possesses, we observed that zinc homeostasis can be regulated by MT-III, reducing the amount of free zinc which can promote Aβ aggregation. Another explanation is that the neuroinhibitory effect of MT-III resulted in neuritic retraction and this prevented Aβ deposition on the cell surface, as initial deposition occurred at the proximal end of the axons and dendrites and has been shown to be dependent on the amount of cell surface available. In this case, hyperactivity and neural sprouting would predispose neurons to Aβ deposition and neurotoxicity.

Conversely, S100β promoted neuronal sprouting and Aβ aggregation which cumulated in a reduced number of viable cells. The action was similar to the pattern observed in cultures coincubated with amyloid and zinc. Furthermore, cells treated with Aβ, zinc and S100β displayed increased neuritic sprouting and higher amounts of Aβ deposition all over the cell and not just on the axonal and dendritic tree.

S100β has also been demonstrated to bind tau. This action occurs prior to neuritic sprouting. In addition, S100β binding was inhibited by tau hyperphosphorylation, as indicated by reduced S100β-tau interaction in AD. This would suggest that the tau-S100β interaction occured prior to the hyperphosphorylation of tau. Furthermore, as tau overexpression and redistribution results in increased hyperphosphorylation, this data could be interpreted as an induction factor culminating in the production of neurofibrillary tangles. In addition to tau binding, the physical requirement of zinc suggested an important role of

zinc-S100β interaction. The metal-binding coincided with aberrant neuritic sprouting and combined with the physical data regarding Aβ deposition would suggest S100β facilitated Aβ-induced neurotoxicity.

Aging is also considered a risk factor for Alzheimer's disease. Without indication of a genetic mutation of one of the FAD genes, AD does not occur until an individual is beyond 60-65 years of age. There is also a positive correlation of age and probability of AD. Through the examination of rats, we observed that there was an increase in the cytokine factors IL-1β, IL-6 and TNFα. These cytokines are believed to have an intimate role in the development of AD. IL-6 upregulates APP expression, while Aβ increases IL-1β production. IL-1β, in turn, increases the amount of S100β produced (Griffin *et al.*, 1988). This would represent a positive feedback loop, whereby cytokine production propagates the deposition of Aβ. In addition to aging and cytokine expression, it was also discovered that astrocytes extracted from aged rats were less effective in protecting PC-12 cells from Aβ-induced toxicity. Whereas astrocytes from pup and adult rats mobilized to form a physical barrier around Aβ aggregates, this activity was not as evident as in the aged group. Furthermore, there was a dramatic decline in the PC-12 population when coincubated with aged astrocytes, suggesting that within the aging process, astrocytes do not produce the same cytokines and this in turn can be directly and indirectly (via Aβ interaction) hazardous to the neuronal population.

7.2 Hypothetical Implications for Alzheimer's Disease

MT-III and S100β are constitutive proteins in the brain. Their roles are predominantly metal-binding and modulation of neuronal sprouting. With aging, and more

evident in Alzheimer's disease, the expression S100β is increased. Furthermore, increases in IL-1ß can induce S100ß expression and decrease MT-III production (Uchida, 1999). In turn this results in two phenomenons: first, there is increased neuritic sprouting; and second, we observe increases in labile zinc. The first action requires an increase in tau, as well as the potential displacement of tau to non-axonal compartments facilitated by S100β. This in turn predisposes the neuron to Aβ toxicity. The increase in free zinc has several implications, including increased excitotoxicity and also promotion of Aβ fibrillization and aggregation. Further to these events is the constitutive aging of astrocytes which leaves them with a reduced protective capacity. This situation is exacerbated in AD where the events of neuronal death are accelerated by hyperphosphorylation causing neurofibrillary tangles and Aβ production and aggregation.

In addition to increases observed in aging, in Alzheimer's disease increases in the levels of IL-1β and IL-6 result in increased amounts of Aβ and S100β (Sheng *et al.*, 1998; Arends *et al.*, 2000; Sheng *et al.*, 1995). Aβ in turn increases IL-1β production (Sheng *et al.*, 1998). Furthermore, via a positive feedback loop, zinc, which can be shuttled via S100β, induces IL-1ß production (Bui *et al.*, 1994; Uchida, 1999). Both zinc and IL-1 can induce metallothionein-I and –II production, but not MT-III. In fact, MT-III production is decreased by IL-1 (Uchida, 1999). While the events of MT-III and S100β may appear distinct, there is a common link via IL-1β and zinc activity. The schematic of events can be seen in Fig 7.1.

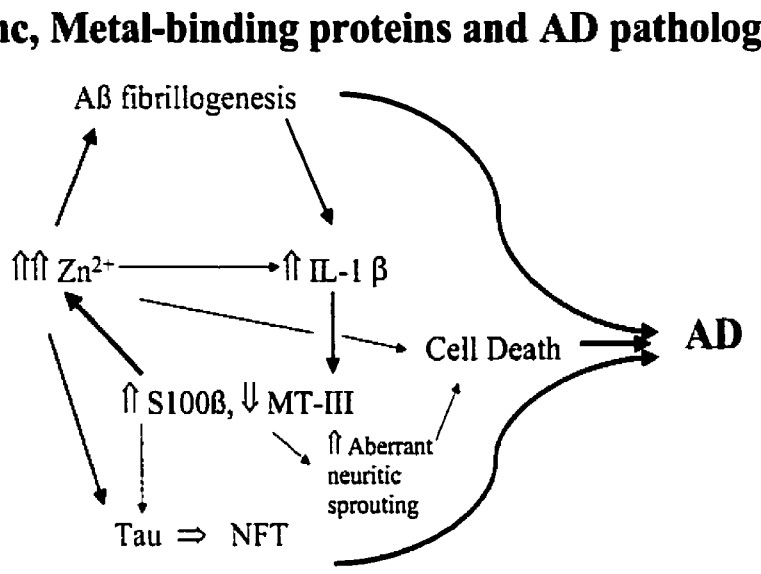

Figure 7.1: Unifying schematic of the alterations observed in Alzheimer's disease and points of modulation by MT-III and S100β.

7.3 Future Experiments

Analysis of cognitive function and metal binding proteins would prove to be a critical experiment in understanding the role of MT-III and S100β in AD. If the function of MT-III is to protect neurons from aberrant neuritic outgrowth, zinc toxicity and zinc-induced amyloid fibrillization and as a disease outcome, Alzheimer's disease, the correlation of MT-III levels with severity of dementia would be useful in implicating whether MT-III expression is a causative or a consequence of the disease process.

In order to test the hypothesis that MT-III downregulation or S100β overexpression modulates neuronal function and can determine its outcome, it is necessary to examine their effects in concert with additional pathogens. This work individually identifies biochemical processes that occur in neuronal-like cell lines with the addition of MT-III or S100β, but a more definitive action may be elucidated through transgenic modelling. Individually, transgenic knockout MT-III mice do not present with any cognitive deficits. S100β transgenic overexpressers have minor memory changes but no detectable pathological alterations. In addition, MT-III null mice may be more susceptible to kainate or glutamate toxicity but do not exhibit any changes until there is a stimulus present. As the APP mouse models have been well characterized, it would be a suitable line to crossbreed with to see if MT-III nulls or S100β overexpressors to determine whether the resultant mice have accelerated plaque formation. In addition, it may be important to see if these plaques contain a higher zinc load. It would also be beneficial to examine neuronal sprouting in these animals and compare to controls and APP alone animals. Measurements can be made based on plaque load, amount of Aβ present in brain, zinc content in plaques, percentage of dystrophic neurites, number of neurons remaining.

As MT-III inhibition and S100β induction may be under IL-1β control, double transgenics of IL-1 and APP mice may be able to demonstrate an upstream effect and determine if the pathological progression is determined by S100β or MT-III activity, or if this action is upstream of these proteins. This would serve as a suitable control experiment to determine whether structural examination of Aβ fibril formation in the presence of zinc can be impeded by MT-III or enhanced by S100β. Aβ undergoes a beta-sheet conformation, which can be induced faster by zinc. This action is prior to aggregation and plaque

formation. Using circular dichroism (CD), it is possible to observe the beta-sheet formation over time. By coincubation of Aβ in a small chamber (see diagram in Fig. 7.2) surrounded by zinc, metallothionein III or S100β. The internal chamber containing Aβ would be in equilibrium with the external chamber, separated by a 1 kD dialysis membrane which selectively permits the exchange of only zinc and the buffer (and not the metal-binding proteins).

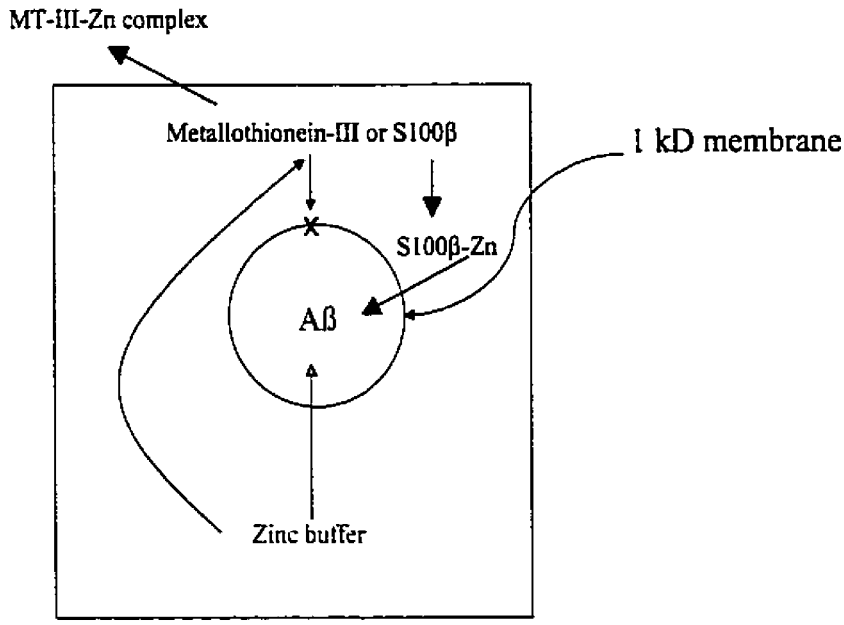

Figure 7.2 Schematic for analysis of zinc induced beta-sheet formation of Aβ in the presence of metallothionein III or S100β. Aβ is placed in a dialysis cup with a 1kD membrane permitting only the buffer and zinc to pass. Aβ samples are periodically removed from the dialysis cup and analyzed using CD. Aβ with buffer alone (control) would undergo β-sheet formation and aggregation in a time-dependent manner. Zinc addition in our model would accelerate the β-sheet formation, whereas MT-III + zinc would be similar to Aβ with just

buffer. S100β would either have the same effect as zinc in buffer. Finally, S100β + zinc presumably would have the fastest Aβ aggregation kinetic.

7.4 Evidence of a Role for MT-III and S100β in the Development of Alzheimer's Disease

Alzheimer's disease is a multifactorial process with, currently, no identifiable initiating event. This work identifies several biochemical processes related to the development of the disease with a theoretical implication on how the disease may develop. First, alterations in cytokine levels in aging may alter the neuroimmunological response in aging. AD is often considered a sped-up process of aging and in this model, the lack of neuroprotective capacity results in greater neuronal toxicity and death. Furthermore, as there is evidence that IL-1β is increased in aging, this may increase the levels of S100β and decrease MT-III. In addition, astrocytes produce IL-1β, as well as S100ß and MT-III.

A multitude of events can occur pending the alteration in the metal binding profile, including increased aberrant neuritic sprouting leading to increased cellular exposure to toxic events such as free radical damage. In addition, there is an apparent alteration in zinc binding. MT-III acts as a reservoir for zinc, whereas S100β, which binds zinc at a lower affinity, may act as a labile reservoir for zinc transport. The facilitation of zinc transport, instead of storage can lead to the development of various events associated with zinc, including direct zinc toxicity, such as glutamate channel activation, zinc-induced Aβ fibrillization, or enhancement (in the presence of S100β) of neuritic sprouting and redistribution of tau from axonal to somatodendritic compartments.

While it may still be possible to conclude that these metal binding proteins do not alter the progression of AD directly, the evidence presented provides a strong case that aging, MT-III and S100ß can play a role in critical neuronal events associated with AD.

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Appendix I: Supplemental to Chapter 3

This appendix contains data not used in the paper in Brain Research Research (Yu, W.H., Lukiw, W.J., Bergeron, C., Niznik, H.B. and Fraser, P.E. (2000) *Metallothionein III is reduced in Alzheimer's disease.* Brain Res. 894:37-45).

These include:

1) Revised Fig. 3.5
2) Table summarizing sex distribution of the AD and control groups used in the Western Analysis. In addition, information is provided on the types of drugs used, duration of AD if known and cause of death.
3) Scatterplot of MT-I/II measurements from Western Analysis in order to establish levels of detection and linearity of measurements.
4) Western blot with purified MT-I/II used in sample to establish a standard used in every set of gels run.
5) Regression analysis of effect of age on MT-III expression.
6) Discussion of Appendix I

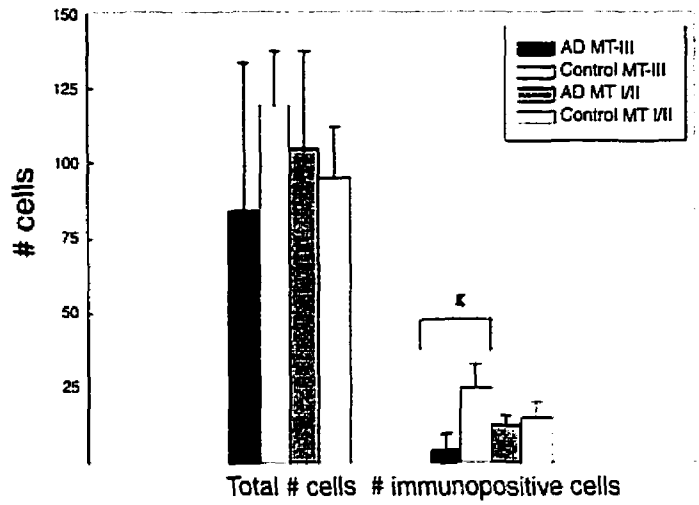

Figure 3.5: Analysis of control and AD brains for immunopositive MT-I/II and –III staining.

Analysis of immunohistochemical staining of MT-III and MT-I/II from brain slices of AD and control temporal brain tissue. For each of the samples, a 250X magnification was used and 20 fields were examined per section to derive a value for both number of immunopositive cells and of total cells per viewing field. Each brain section used was given a value based on total immunopositive and total number of cells present divided by the number of counts (10) made. An average value was derived from the three samples analyzed per group. Based on the Student's T-test, MT-III immunoreactivity is significantly decreased ($p<0.01$) in AD tissue compared to control sample, as indicated (*).

Table I.1: Summary chart of autopsy reports from samples used in Western analysis of MT-I/II and MT-III. The average age of the AD group was 75.48 +/- 10.22 years, while the control group had a mean age of 70.63 +/- 18.00 years. The sex distribution was 56% female and 44% male in the AD group, while there were 46% female and 54% male in the control group. Duration of AD ranges from 1-20 years in this group, though AD is not confirmed until autopsy. Most patients in the AD group died of bronchopneumonia, though general organ failure was the primary cause. Previous illnesses and medication usage was also diverse in both groups. Cancer and infection were the two most common ailments listed in both groups. Furthermore, antibiotics, thyroxin replacements, renal drugs and anti-inflammatory drugs were the most common medications taken in both groups.

AD

Sample	Age at Death	Sex	# yrs of diagnosed AD	Cause of Death	Drugs Used
816	76	M	5	Not listed	Amoxil, serax, colace
826	85	M	3	Bronchopneumonia	Largactil, nozinan, amoxil, ASA
827	83	F	3	Not listed	Gravol, vitamins
845	65	M	4	Bronchopneumonia	Dilantin, naproxen, prednisone, haldol, gravol, largactil, vlaium, bactrium, urecholine, pyridium, dyazide
891	74	M	8	Bronchopneumonia	Antibiotics
1142	89	F	5	Pneumonia	Clonazepam, haldol, dilantin
1144	70	M	1	Diabetes, cardiac failure	Not listed
1145	78	M	6	Bronchopneumonia	Not listed
1197	62	M	10	Seizures, bronchopneumonia	Synthroid, melcaril, haldol, amantadine
1203	88	F	Not listed	Heart failure, renal infection	None
1210	83	M	Not listed	Pulmonary congestion	Digoxin (acute)
1215	87	F	7	Not listed	ASA, Eltroxin, synthroid, +others
1278	58	F	14	Seizures	Unknown
1283	60	F	20	Bronchopneumonia	Haldol, cogentin, rivotril
1289	79	M	8	Not listed	Zantac, alzene, colace prednizone
1297	75	F	3+	Heart failure	Thyroid , heart drugs
1302	80	F	6	Cardiorespiratory failure	Losec, palafer, fuarate
1304	91	F	Post mortem	Not listed	Not listed
1312	69	F	Not listed	Not listed	Not listed
1321	82	F	10	Necrotic bowel	Haldol, reserpine, diuretic
1337	66	F	12	Respiratory failure	Not listed
1338	58	M	2	Bronchopneumonia	Not listed
1339	84	F	3+	Congestive heart failure	None
1350	80	F	Not listed	Not listed	Bromocriptine, elavil, chlorohydrate
1375	65	M	6	Bronchopneumonia	Not listed

CONTROL

Sample	Age (Death)	Sex	Cause of Death	Drugs Used
919	60	M	Suicide	Tylenol, ventolin, Theodor
975	77	F	Cerebellar hemorrhage	Beta-blocker
1084	46	F	Heart failure	Not listed
1190	92	F	Tachycardia, heart attack	Lasix, quinidine
1214	92	M	Heart attack	Lasix, venotlin, ativent, lorazapam
1244	92	M	Cancer; cerebral infarcts	Dilantin, lasix, zantac, furosomide, ranitidine, cipro, novasen, entrophn
1271	57	M	Cancer	Prednisone, fluoracil, epiburinm cyclophosphamide, morphine, atropine, lorazepam, percocet
1280	77	M	Arteriosclerosis	Lidocaine, atropine, epinephrinem quinidine, digoxin, capoten, coumadin, lasix, tagamet
1292	41	M	Drowning; head trauma	Not listed
1342	73	F	Cancer; immunodeficiency	Not listed
1361	70	F	Cancer	Not listed

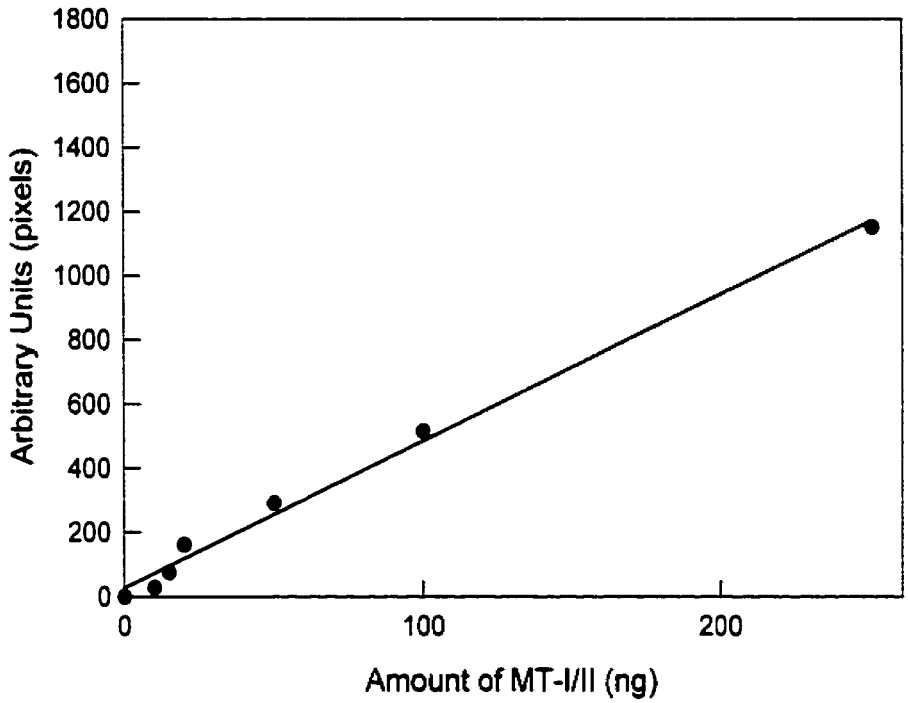

Fig I.1: Linear regression of commercial MT-I/II samples via Western blotting using 10-250ng of purified protein. Sample detection is linear (R^2=0.982) within this range.

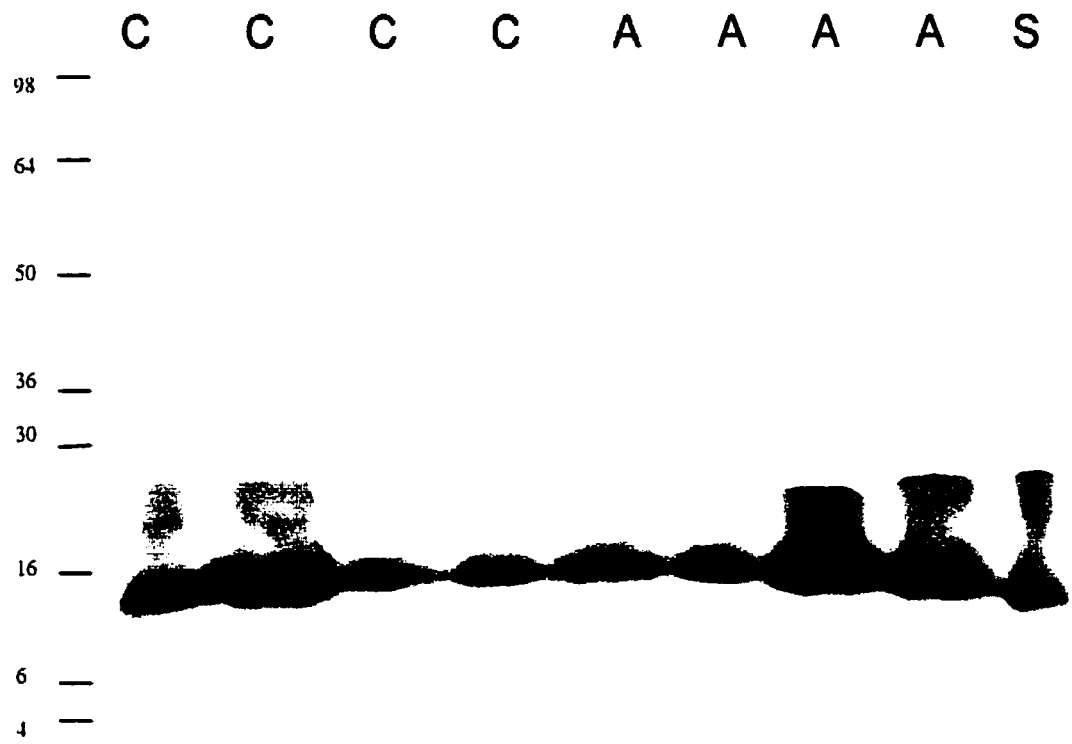

Fig. I.2: Western analysis of MT-I/II of 100μg of four control (C) and four AD (A) brain homogenate samples along with 100 ng of commercially available MT-I/II purified protein. 100 ng of commercial MT-I/II was used in every time a group of samples were run to control for inter-gel variations.

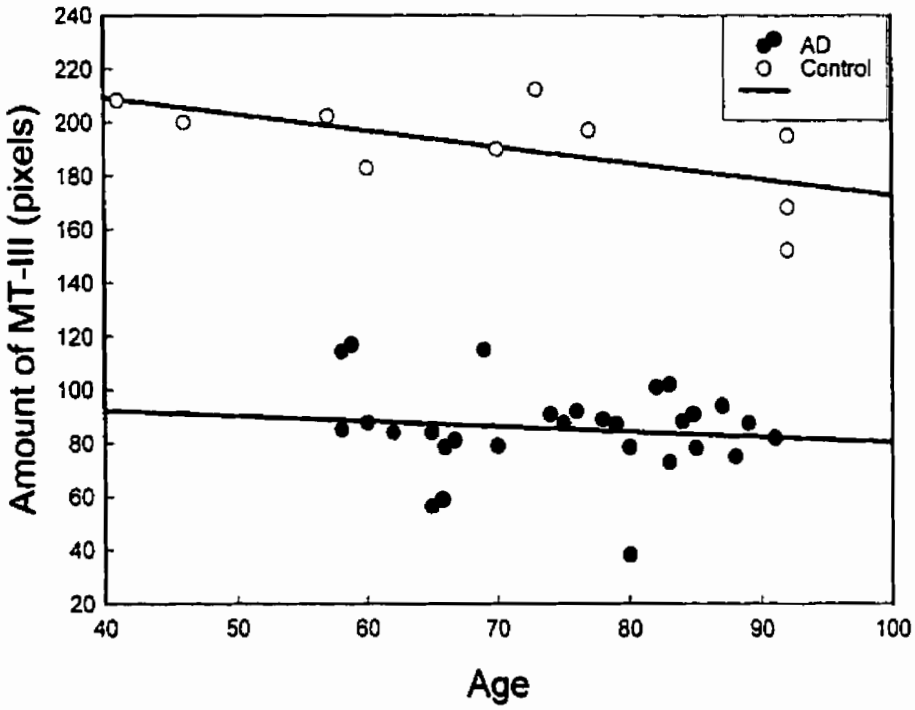

Fig. I.3: Scatterplot of MT-III in brain versus age with linear regression analysis of AD and control groups. The MT-III levels in AD samples have a moderate slope of -0.194, while the control group's slope is –0.64. AD samples have a linear regression of R^2=0.15, while control has a linear regression of 0.38. In both groups there is a non-significant correlation of age with decline in MT-III levels.

I.1 Discussion for Appendix I

I.1.1 Explanations for MT-III reduction in Temporal Cortex of AD brains

I.1.1.1 IL-1ß

Other studies investigating MT-III levels in AD have failed to derive the mechanism for its downregulation. MT-III decrease in AD is possibly a result of an increase in IL-1*ß*, which has been shown to be increased in Alzheimer's disease (Sheng *et al.*, 1996b; Griffin *et al.*, 1994). Uchida (1999) found that stimulation of IL-1ß and not other cytokines resulted in a decrease in MT-III levels. IL-1ß induction can be achieved through various mechanisms, including induction of inflammation pathway and increased free zinc levels.

I.1.1.2 Medication

Other factors that may affect MT-III and protein levels in general include drugs, illness and types of illness. In order to assess these implications, the experimental groups used in the Western analysis were identified by years of diagnosed AD, causes of death and drugs used prior to death. Among the latter, for those with medications listed in the autopsy reports, there is considerable overlap of drugs taken. Consequently, it is unlikely drugs play a major role in determining MT-III levels. Had there been a discrepancy between groups, for instance, the AD group did not take anti-inflammatory medication, resulting in increased IL-1ß expression and consequently decrease MT-III levels, than this would have been a critical factor. In this sample group, a majority of either group did not consume antibiotics.

I.1.1.3 Illness and Cause of Death

Another complication may be years of infirmary, as well as whether the cause of death was sudden or prolonged. Decreased metabolic function or reduced oxygen supply may alter protein expression. AD patients died mostly of bronchopneumonia, though, there are cases of heart or other organ failure. Control patients in this study died from cancer or heart attack, which may have been sustained after prolonged periods of arteriosclerosis. Duration of illness may also play an important role in protein regulation, this variable is not consistent in this study. Overall, there appears to be no fixed length of illness or infirmary, it is possible that disease state and length of illness can alter the MT-III levels. Accordingly, if this was evident, then MT-III levels in AD could be reduced by this event.

I.1.2 Establishing limits of detection and trends in sample group

In order to establish various limits of detection, several experiments not included in Chapter 3 are illustrated in this Appendix. As shown in Chapter 2, the limit of MT detection can be as low as 10ng. In this study, it was established that approximately 100ng of MT-I/II would be analyzed, which in Fig. I.1 is within the linear range of detection of MT protein. Furthermore, in each Western blot of MT-I/II, a commercially available MT-I/II protein derived from horse liver and kidney (Sigma) was used. In order to nullify inter-gel variation, all samples were standardized based on the relative quantity of this standard. The major band identified in this study was derived from the dimerized form at approximately 13-14kD. A monomer was observed at approximately 6-7kD, but this sample was inconsistent and difficult to quantify as it was below the limits of detection. Higher molecular weight bands are common to the antibody used. While they may be polymers of the protein, it has not

been suggested by previous analysis. In addition, there are two other possibilities as for what they may be. The first is that this protein may have non-specific binding to other proteins in the brain homogenate and second, increased proteins detected as a result of protein fixation on the membrane using glutaraldehyde. In order to resolve this problem, it might be necessary to sequence some of these bands to identify if they contain metallothioneins.

Finally, in order to establish that MT-III expression was not age-dependent. MT-III expression was plotted against age. In both control and AD groups there is a modest negative slope, that had very low regression levels (R^2=0.15 for the AD group and R^2=0.38 for the control group). This lack of consistency suggests that aging is not a factor in MT-III levels.

These additional figures illustrate that age, drugs and intersample variation are not likely to be factors affecting the outcome of this study. Factors that may alter MT-III levels include IL-1ß expression, which is increased in aging, and even more dramatically in AD. In addition, disease state and cause of death should not be ruled out as factors involved in MT-III reduction in AD.

APPENDIX II:

Letter from Publishers and Authors for Release of Publication

FCR/smc/February 2001.023
2 February 2001

Dr Haung Yu
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Elsevier Science

Dear Dr Yu

JOURNAL OF BIOCHEMICAL AND BIOPHYSICAL METHODS, Vol 32, 1996, Pages 77-83, Mizzen et al

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University of Virginia
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January 24, 2001

To Whom It May Concern:

Regarding the article entitled "Sensitive detection of metallothioneins-1, -2 and 3 in tissue homogenates by immunoblotting: a method for enhanced membrane transfer and retention.", authored by Craig A. Mizzen, Nicholas J. Cartel, Wai Haung Yu, Paul E. Fraser and Donald R. McLachlan, that appeared in the Journal of Biochemical and Biophysical Methods (1996) 32:77-83.

Wai Haung Yu was responsible for generating the antiserum to metallothionein-3 that was used and contributed to the work by performing some of the immunoblot experiments shown. He also helped prepare the manuscript and reviewed it prior to submission.

Sincerely,

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FCR/jj.feb01.222
23 February 2001

Haung Yu
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APPENDIX III:

Publications Not Included in Thesis

Additional Books

Editor-in-Chief, Pressacco, J and **WH Yu. Pharmacological Treatment of Diseases.** Toronto: Urban Angel Press, 1996.

Additional Publications

1) Levesque, G, Yu, G, Nishimura, M, Zhang, DM, Levesque, L, **Yu, H**, et al. Presenilins interact with armadillo proteins including neural-specific plakophilin-related proteins and ß-catenin. **J Neurochem** (1999) 72: 999-1008.
2) Yu G, Nishimura M, Arawaka S, Levitan D, Zhang L Tandon A, Song YQ, Rogaeva E, Chen F, Kawarai T, Supala A, Levesque L, **Yu H**, *et al.* (2000) Nicastrin modulate presenilin-mediated *Notch/GIp-I* signal transduction and βAPP processing. **Nature** (2000 Sep 7) 407(6800): 48-54.

Additional Submissions

1) Chen, F, Yu, G, Arawaka S, Nishimura, M, Kawarai, T, **Yu, H**, Tandon, A, Supala, A, Son, YQ, Rogaeva, E, Milman, P, Sato, C, Zhang, L, Fraser, PE and St George-Hyslop, PH. Nicastrin binds to membrane-tethered *Notch*. Nature Cell Biology (2000).

Additional Publications in Preparation

1) Morrison, MR, **Yu, WH**, Go, L and J McLaurin. Peroxisome proliferating activator receptor expression and activity during aging, in prep.
2) Tandon, A, **Yu, WH**, Nishimura, M, Yu, G, Fraser, PE and PH St George-Hyslop. Absence of presenilin 1 and nicastrin at the surface, in prep.

3) Tandon A, **Yu, WH**, Rogaeva, E, Sato, C, Kawarai, T and PH St George Hyslop. P25 levels are not specifically elevated in Alzheimer's disease relative to control brains or other neurodegenerative disorders, in prep.

4) Yang, DS, **Yu, WH**, Lieu, F, Karunaratne, A, Duthie, M, Deng, J, Bergeron, C, Rozmahel, R, Westaway, D, Mount, H, Hyslop, P and PE Fraser. Characterization of the presenilin associated protein, neural plakophilin-related armadillo protein (NPRAP), in prep.

Additional Abstracts

1) **Yu, WH**, Janus, C, Pearson, J, Heslin, D, Haight, J, Parisen, K, Renlund, R, Bergeron, C, St George-Hyslop, P, Westaway, D. Immunization with beta amyloid peptide attenuates cognitive impairment of Tg APP_{CRND8} mice at early stages of immunization. New Orleans: **Society for Neuroscience.** Nov 4-9, 2000.

2) Tandon, A, **Yu, WH**, Nishimura, M, Yu, G and PE Fraser. Absence of cell surface presenilin in HEK293 and MDCK cells. Washington: **World Alzheimer Congress.** July 8-13, 2000.

3) Yang, DS, **Yu, WH**, Lieu, F, Karunaratne, A, Duthie, M, Deng, J, Mount, H and PE Fraser. Characterization of the presenilin associated protein, neural plakophilin-related armadillo protein (NPRAP). Washington: **World Alzheimer Congress.** July 8-13, 2000.

4) **Yu, WH,** Kruck, TPA, Papaioannou, ND, and DR McLachlan. Inhibition of toxic desferrioxamine metabolism by isoniazid. Minneapolis: **Fourth International Conference on Alzheimer's Disease and Related Disorders**. July 29th - August 3rd, 1994.

5) Papaioannou, ND, **Yu, WH,** Krishnan, B, McLachlan, DRC, and TPA Kruck. Effect of Iron on aluminum chloride uptake in rabbits. Minneapolis: **Fourth International Conference on Alzheimer's Disease and Related Disorders**. July 29th - August 3rd, 1994.

6) **Yu, WH.** Characterization and Inhibition of Desferrioxamine Metabolism. Toronto: **Pharmacology Research**. April 6th, 1992.