CYTOSKELETAL PROTEIN DYSFUNCTION AND OXIDATIVE MODIFICATION IN ALZHEIMER'S DISEASE

By

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This work is dedicated to

my parents,

Anthony and Sheryl Boutté,

to my uncle and aunt,

Mickey and Linda Clarke,

to Norick “Pops” Bizé,

& to the memories of Emma and Cassandra

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Aβ: amyloid beta peptide

AA: arachidonic acid

AAPH: (2-amidino- propane) dihydrochloride

AD: Alzheimer's Disease

AGE: advanced glycation endproduct

ALS: Amyotrophic Lateral Sclerosis

APP: amyloid precursor protein

BA: buffer A

CNS: central nervous system

C-terminal: carboxy-terminal

DHA: docosohexanoic acid

DI/FS: detergent insoluble and formic acid soluble

DRG: dorsal root ganglion (cell)

eNOS: endothelial NOS

FTDP-17: fronto-temporal dementia with Parkinsonism linked to chromosome 17

GC: gas chromatography

GFAP: glial fibrillary acidic protein

GSK-3β: glycogen synthase kinase isoform 3β

GTP: guanosine tri-phosphate

H: helix

H_2O_2: hydrogen peroxide

HHE or 4-HHE: 4-hydroxy-2-hexenenal

HNE or 4-HNE: 4-hydroxy-2-nonenal

HNE(AC)3: HNE tri-ester

iNOS: inducible NOS

IsoP: isoprostane

LC-LC: two dimensional electrophoresis

LCM: laser capture microscopy

LOAD: late onset AD

LPO: lipid peroxidation

LPS: lipopolysaccharide

MALDI: matrix assisted laser desorption ionization

MAP(s): microtubule associated protein(s)

MCI: mild cognitive impairment

MFG: mid frontal gyrus

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MS-MS: tandem mass spectrometry

MT: microtubule

NADPH: nicotinamide adenine dinucleotide phosphate, reduced form

NeuroP: neuroprostane

NF: neurofilament

NFT: neurofibrillary tangle

NGF: nerve growth factor

NMDA: N-methyl-D-aspartate

nNOS: neuronal nitric oxide synthase protein

NO: nitric oxide

$O_2^{\bullet-}$: superoxide

ONOO-: peroxynitrite

PHF: paired helical filament

PMI: post mortem interval

PS2: presenilin 2 gene mutant

P-Tau: phosphorylated tau

RNA: ribonucleic acid

RNS: reactive nitrogen species

ROS: reactive oxygen species

SALSA: scoring algorithm for spectral analysis

sAPPα/β: secreted APP α/β

SMTG: superior middle temporal gyrus

swAPP: APP Swedish mutant

Tg: transgenic

TOF-TOF: tandem time of flight

CHAPTER I

BACKGROUND

1.1 Introduction

Alzheimer's disease (AD) is the most common form of age-related dementia. Western populations are 'aging'; therefore, AD is poised to afflict up to 12 million Americans by the year 2020. The neurodegeneration in Alzheimer's disease is associated with an early increase of oxidative damage (Nunomura, Perry et al. 2001), amyloid beta (Aβ) peptide deposition in senile plaques, and tau accumulation neurofibrillary tangles (NFTs). These pathological hallmarks are found primarily in frontal, temporal, and parietal cortices as well as in the hippocampus.

Several risk factors for familial AD exist. Inheritance of the apolipoprotein *E4* (*APOE4*) allele among the common alleles *APOE2,3,4*, is associated with increased risk of developing AD (Roses 1998; Tsuang, Larson et al. 1999). Mutations in amyloid precursor protein (APP) or Presenilins (PS) 1 and 2 cause rare autosomal dominant forms of AD (McPhie, Lee et al. 1997; McPhie, Golde et al. 2001). Familial and late onset AD (LOAD) share common endpoints of oxidative damage, plaques, tangles and neurodegeneration; however, LOAD accounts for more than 90% of AD cases. The links between oxidative damage, microtubule (MT) protein modification, axonopathy and their role(s) within the

discreet etiology and mechanisms in LOAD pathogenesis are not fully understood.

In the present studies, I have tested the hypotheses that reactive products of lipid peroxidation (LPO) are the effectors that lead to neuronal MT (MT) collapse and inability to polymerize in cells and that this same loss of MT function is recapitulated in human brain tissue from LOAD, frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), but not mild cognitive impairment (MCI). MT dysfunction in human brain tissue parallels the increasing abundance of modifications induced by oxidative damage of MT proteins that is also in accordance with protein insolubility. A proposed scheme of AD pathogenesis, adapted from a recent review by Drs. Hardy and Selkoe, is presented in the flow chart below (Figure 1).

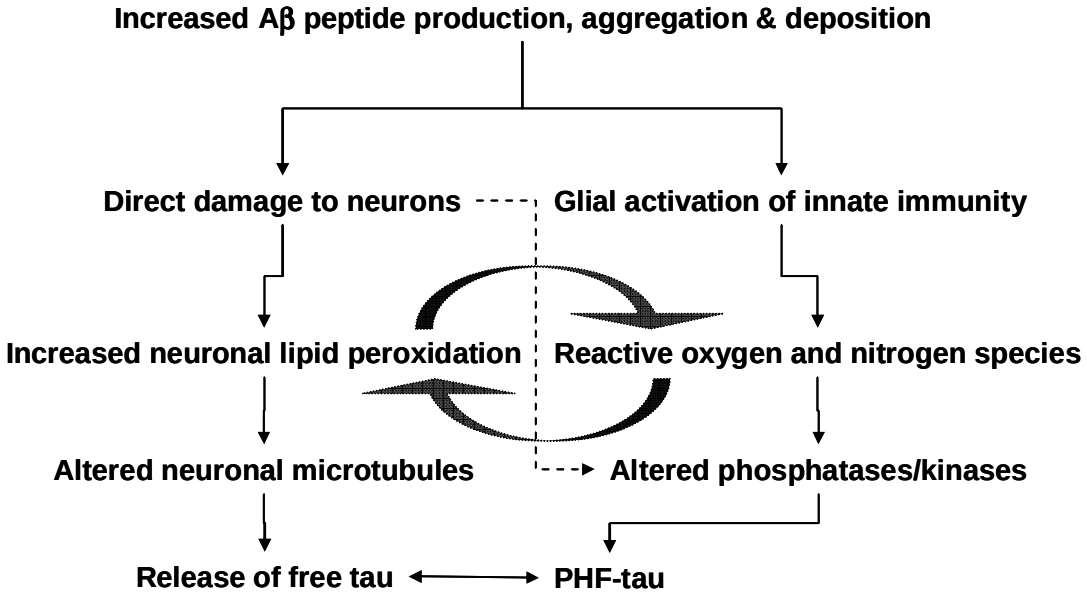

Figure 1. Model of AD pathogenesis adapted from Hardy and Selkoe.

Amyloid beta (Aβ) peptide production, aggregation and deposition into neuritic plaques may directly damage neurons or activate innate immunity and lead to increased neuronal LPO and robust increases in reactive oxygen and nitrogen species (ROS and RNS) from glial cells. ROS, RNS and LPO are part of the milieu of self-sustaining and ongoing free radical oxidation mechanisms which only end when all available substrates, protein nucleic acid and lipid, are consumed. This oxidative damage and neuronal damage alters MTs, which are composed of tubulin heterodimers and MT associated proteins (MAPs). The phosphatases and kinases that modulate tau, a MT stabilizing MAP, are also affected and leave tau hyperphosphorylated and with a much weaker affinity for tubulins. Free, hyperphosphorylated tau easily self-associates, creating an intra-neuronal inclusion characteristic to AD pathology, the NFT.

1.2 Clinical Manifestation of Alzheimer's Disease

AD is a progressive and fatal neurodegenerative disorder manifested by cognitive and memory deterioration, progressive impairment of activities of daily living, and a variety of neuropsychiatric symptoms and behavioral disturbances. The five areas that require assessment (and periodic reassessment) in the patient with AD are daily function, cognition, co-morbid medical conditions, disorders of mood and emotion, and caregiver status (Cummings and Jeste 1999; Chung and Cummings 2000; Cummings 2000; Cummings and Cole 2002; Cummings, Frank et al. 2002).

Functional and behavioral disturbances are characteristic of the disease. Patients progress from the loss of higher-level activities of daily living, such as check writing and the use of public transportation, through abnormalities of basic activities of daily living, such as eating and grooming, as the disease enters advanced phases (Galasko, Bennett et al. 1997). Behavioral disturbances also progress over the course of the illness (Kawas 2003). Mood change and apathy commonly develop early and continue for the duration of the disease. Psychosis and agitation are characteristic of the middle and later phases of the disease (Mega, Cummings et al. 1996).

The typical clinical syndrome of AD includes an amnesic type of memory defect with difficulty learning and recalling new information, progressive language disorder beginning with anomia and progressing to fluent aphasia, and disturbances of visuospatial skills manifested by environmental disorientation and difficulty copying figures in the course of mental status examination (Cummings and Benson 1986). There are usually deficits in executive function (planning, insight, judgment) and the patient is typically unaware of memory or cognitive compromise. All cognitive deficits progressively worsen. Neuropsychiatric symptoms are common in AD. Apathy is apparent early in the clinical course with diminished interest and reduced concern. Agitation becomes increasingly common as the illness advances and is a frequent precipitant of nursing home placement. Depressive symptoms are present in about 50% of patients and approximately 25% exhibit delusions (Mega, Masterman et al. 1999). Motor system abnormalities are absent in AD until the final few years of the disease;

focal abnormalities, gait changes, or seizures occurring early in the clinical course of dementia make the diagnosis of AD unlikely. Patients with AD usually survive 7 to 10 years after onset of symptoms and typically die from bronchitis or pneumonia (McKhann, Drachman et al. 1984; Bracco, Gallato et al. 1994).

1.3 Neuropathology

In AD, six developmental stages can be distinguished on account of the predictable manner in which the neurofibrillary changes spread across the cerebral cortex. The pathologic process commences in the trans-entorhinal region (clinically silent stages I and II), then proceeds into adjoining cortical and subcortical components of the limbic system (stages III and IV - incipient AD), and eventually extends into association and primary sensory areas of the neocortex (stages V and VI - fully developed AD) (Braak, Braak et al. 1996; Braak, Griffing et al. 1999). Beginning in predisposed induction sites in the allocortex, the lesions follow a predictable sequence as they engulf other territories of the cerebral cortex and a specific set of subcortical nuclei. Some components of the brain are devastated, while others remain intact until the end phase of the disease. Assessment of the location of the afflicted neurons and the severity of the lesions allows the distinction of stages in the development of the disease. The degenerative process begins with the emergence of the first lesions, at whatever age it occurs. The illness remains subclinical for years and proceeds inexorably, gradually laying waste to higher order limbic system centers. Clinical symptoms are observed only late in the course of the disease,

and their appearance is usually concurrent with the encroachment of the destructive process upon neocortical association areas.

As mentioned earlier, AD is a syndrome with several monogenetic autosomal dominant causes, each related to metabolism of the APP and its cleavage by β and γ secretases to form amyloid β (Aβ) peptides. However, the much more common form(s) of AD does not have a single genetic cause. This form, referred to as sporadic AD, likely derives significant input from the intertwined processes of aging, inherited susceptibilities such as the *E4* allele of the apolipoprotein gene (*APOE4*), and environmental factors (Khachaturian, Corcoran et al. 2004). A dominant hypothesis is that all forms of AD share increased production or accumulation of Aβ peptides. Neuritic plaques are a pathological hallmark of AD. Neuritic plaques are extracellular deposits of Aβ peptides, composed mostly $A\beta_{40}$ and $A\beta_{42}$ as well as other less abundant Aβ peptides. These plaques are surrounded by dystrophic axons and activated astrocytes and microglia (Cummings, Su et al. 1992). In AD, this peptide forms detergent- insoluble aggregates and smaller soluble aggregates: oligomers or amyloid-derived diffusible ligands (ADDLs) and protofibrils (Klein 2002). The precise mechanisms of Aβ mediated neurodegeneration are not yet clear. One proposal is the activation of innate immune response from microglia and astrocytes by extracellular Aβ peptide aggregates (Hu, Akama et al. 1998). Another is that Aβ peptides are directly toxic to neurons. Either directly or indirectly, the accumulation of Aβ peptides is thought to be the major stimulus of increased oxidative stress in AD.

APP is a normally expressed protein that has a role in neurogenesis by involvement in growth cone formation during development. Growth cones must extend to form synaptic connections. These cones are mainly composed of tubulins, the proteins that form the MTs. Therefore, even during development, the cytoskeletal network and APP or its cleavage products, Aβ, secreted amyloid precursor proteins α and β (sAPPα and aAPPβ) are intrinsically linked. Overproduction of $A\beta_{40}$ and $A\beta_{42}$, which is more toxic and more apt to form fibrils, is caused by multiple genetic mutations of the APP or presenilin genes in familial AD (Dumery, Bourdel et al. 2001). However, the mechanism behind the accumulation of Aβ peptides in LOAD, which is more than 90% of all AD cases is more elusive. Regardless, the general consensus is that Aβ accumulation leads to oligomers and then larger aggregate formation. These aggregates are detergent insoluble. Other proteins in AD share these characteristics and are in accordance with disease progression, specifically tau protein.

The other pathological hallmark of AD is NFT formation and, its ultrastructural correlate, disruption of neuronal MTs. NFTs are composed primarily of paired helical filament PHF-tau. Tau is a microtubule-associated protein (MAP) that is abnormally phosphorylated and modified by reactive oxygen and nitrogen species in AD brain and thereby aggregates into PHF-tau. How tau becomes abnormally modified is not clear but a proposed pathogenic sequence of events is that some stimuli disrupt neuronal MTs with release of tau that in combination with altered activity of kinases and phosphatases and increased oxidative stress leads to abnormally modified tau that aggregates into

PHF-tau. One hypothesis is that reactive products of lipid peroxidation are the stimuli that contribute to neuronal MT collapse and release free tau.

In this regard, MTs and tau have received attention in LOAD (Zhang, Higuchi et al. 2004; Iqbal, Alonso Adel et al. 2005). Several morphologic and immunohistochemical studies have demonstrated changes consistent with MT dysfunction in LOAD, including synaptic vesicles that fail to reach the terminal compartment, vesicle accumulation in neuron soma, and increased mitochondrial elements in lysosomes (Scheff, DeKosky et al. 1990; Praprotnik, Smith et al. 1996; Terry 1996; Hirai, Aliev et al. 2001; Stokin, Lillo et al. 2005). Moreover, a few investigators have noted a reduction in stable MTs in brain specimens from patients with LOAD, even specimens obtained by biopsy relatively early in the course of disease (Paula-Barbosa, Tavares et al. 1987; Hempen and Brion 1996; Cash, Aliev et al. 2003).

Tau was initially identified as the major component of NFTs, filamentous protein aggregates. In normal human brain, tau is encoded by one gene on chromosome 17 that results in 6 alternatively spliced RNA variants that encode exons 2, 3, and 10. Exons 2 and 3 encode part of the N-terminal projection domain that allows tau to interact with the neuronal membrane and other cytoskeletal elements, and exon 10 splicing determines the presence of a 3 or 4 MT binding repeat in the intact tau protein. All three regions, the N-terminal projection domain, the proline-rich region, and the C-terminal MT binding domains are integral to MT function and the latter 2 domains are susceptible to hyperphosphorylation. Although normal tau function is regulated by kinases and

phosphatases to mediate on-and off- MT binding and fluid/labile interactions, hyperphosphorylation is hypothesized to be the seeding mechanism initiating stepwise NFT formation across all neurodegenerative tauopathies (Buee, Bussiere et al. 2000).

NFTs are found in AD, amyotrophic lateral sclerosis/parkinsonism–dementia complex of Guam, corticobasal degeneration, Down syndrome, parkinsonism, progressive supranuclear palsy, and sometimes in Pick's disease. Tau inclusions have also been detected in normal aging in the absence of clinically defined dementia (Delacourte, David et al. 1999; Buee, Hamdane et al. 2002; Sergeant, Delacourte et al. 2005).

Hyperphosphorylated MT-associated tau proteins are the main components of the aggregated filaments found in NFT in AD, Tauopathies are dependent upon the tau hyperphosphorylation and aggregation caused by multiple possible mutations that make tau more susceptible to a change in tertiary structure and open to hyperphosphorylation in autosomal dominant neurodegenerative tauopathies, e.g., FTDP-17. LOAD exhibits no tau mutations although it has shared hyperphosphorylation (Iqbal, Alonso et al. 1993; Alonso, Grundke-Iqbal et al. 1996; Iqbal, Alonso Adel et al. 2005). Like Aβ plaque formation, tau aggregation into NFTs is not due to mutations in sporadic AD. Many hypotheses fault kinases such as GSK3-beta in AD, although there is no genetic link in LOAD.

Hyperphosphorylation of tau leading to NFT formation is the major determinant of tau aggregation in LOAD and other tauopathies. Tau has more

than 79 putative phosphorylation sites that are present within the MT binding regions. After extensive, abnormal, phosphorylation, the resulting modified protein is referred to as paired helical filament (PHF) tau. Tau is also a substrate for modification by ubiquitination, advanced glycation endproducts (AGE), redox potential, polyanion or lipid interactions and lipid peroxidation (LPO) products. Tau is also directly modified by reactive oxygen and nitrogen species (ROS and RNS). All of these non-physiological post-translational modifications or chemical adducts have a role in seeding tau leading to NFT formation. In addition, ROS and RNS are key factors in MT protein insolubility and dysfunction, discussed further later. NFTs are preferentially observed in large pyramidal cells of the hippocampus and entorhinal cortex; both are substructures of the temporal lobe, and are especially susceptible in AD.

1.4 Protein Insolubility Among Neurodegenerative Diseases

A dominant biochemical feature of AD is the accumulation of detergent-insoluble (DI) proteins that can be extracted by highly chaotropic means, such as partial solubility in formic acid (FS). Two proteins of which large proportions make this transition from normal solubility to DI/FS are Aβ and tau, the major protein constituents of senile plaques and NFTs in AD. $A\beta_{42}$ is the main component in senile plaques and the key player or model peptide in the oligomeric aggregation hypothesis in which peptides or proteins change conformation from α helical to β sheet structures and create increasingly insoluble protein aggregates (Trzesniewska, Brzyska et al. 2004).

Despite the importance of this transition of a subset of proteins from normal solubility to DI/FS, relatively little is known about the mechanisms that underlie this change. Recent elegant studies have demonstrated biophysical mechanisms by which Aβ forms insoluble fibrils in vitro, and it seems likely that inherent properties of some protein structures and protein-protein interactions will be key to determining which proteins transition to abnormal and perhaps pathologic insolubility. Another proposed mechanism for DI/FS is post-translational modification of protein by oxidative damage. Indeed, oxidative damage to protein, lipid, and nucleic acids has been repeatedly associated with diseased regions of brain from patients who died with AD and cerebrospinal fluid from patients with early AD and even patients with mild cognitive impairment, a prodromal condition that commonly progresses to AD.

1.5 Microtubule Impairment and Neurodegeneration

Synapse loss and dying-back of axons are characteristic of neurodegeneration (Schaumburg and Spencer 1979; Ball 2003; Mandelkow, Stamer et al. 2003; Stokin, Lillo et al. 2005). Synapse loss occurs early in the disease and correlates with the incipient loss of memory and brain functions leading to the hypothesis that one of the neurobiological defects in AD is a failure of neuronal plasticity (Buee, Bussiere et al. 2000). For example, levels of neuronal plasticity and synaptic proteins are decreased in frontal association cortex areas with high NFT density in AD brains (Callahan and Coleman 1995). This impairment in the axons is also demonstrated by the disruption of

axoplasmic flow (Terry 1998), which is a primary function of the MTs. There is also some evidence that axonopathy is caused by or occurs after Aβ accumulation and neuritic plaque deposition. Using a transgenic mouse model that overexpresses Aβ, Tg-swAPP mice, a recent study determined that cholinergic axons were often unusually large, irregular and were immunoreactive for phosphorylated tau (Stokin, Lillo et al. 2005). Furthermore, these impaired axons contained large numbers of organelles and vesicles, were not myelinated, and were not associated with postsynaptic densities. A growing body of evidence indicated that tau and tubulin dysfunction preceded synapse loss independent of Aβ (Zhang, Higuchi et al. 2004). An abundance of tau alone blocked MT trafficking of organelles and vesicles (Stamer, Vogel et al. 2002).

The events that may trigger axonopathy are glial mediated oxidative stress, production and accumulation of toxic Aβ peptides, and the hyperphosphorylation of tau protein. All of these features may develop gradually and appear to precede the more overt pathological changes in the brain, such as deposition of protein aggregates in the form of amyloid plaques and NFTs. Oxidative damage leads to morphological and functional changes in axons. After exposure to hydrogen peroxide (H_2O_2), one of many products of oxidative damage, axonal swelling and axonal beading developed. No beading was observed in glial fibrillary acidic protein (GFAP)-positive astrocytes, indicating that the effect is specific to neurons (Roediger and Armati 2003).

Since neurons are highly elongated cells, they depend on an efficient transport system for delivering proteins, lipids and other cell components from

the cell body to the synapse. This system is based on MTs which serve as tracks, motor proteins which represent the engines, vesicles and organelles which are the cargoes, and MAPs which serve as ties for the stabilization of the MT tracks. In axons, tau protein is one of the predominant MAPs. It stabilizes MTs and promotes neurite outgrowth. This apparently beneficial role of tau contrasts with its anomalous behavior in several neurodegenerative diseases, most prominently AD, where it occurs in a highly phosphorylated form, detaches from MTs, and aggregates. It has been hypothesized that the detachment of tau from MTs is caused by some imbalance in intracellular signaling which favors excessive phosphorylation (Alonso, Zaidi et al. 1994; Alonso, Grundke-Iqbal et al. 1996; Iqbal and Grundke-Iqbal 1996; Iqbal, Alonso et al. 1998; Trzesniewska, Brzyska et al. 2004; Iqbal, Alonso Adel et al. 2005). This in turn would detach tau from MTs, prompt their decay, so that axonal transport would be interrupted, while the detached, soluble tau would aggregate and thus cause a generalized clogging of cytosolic space. It is not clear whether detachment of tau and its aggregation and the breakdown of MTs are the early causes of degeneration or the later consequences of Aβ neurotoxicity and plaque formation.

1.6 Microtubule Structure and Function

MTs are essential cytoskeletal polymers that are made of repeating α/β-tubulin heterodimers and are present in all eukaryotes. MTs affect cell shape, cell transport, cell motility, and cell division. All of these functions involve the

interaction of MTs with a large number of MAPs, which are important for the regulation and distribution of MTs in the cell.

In addition to well studied motor proteins such as kinesin and dynein, tau protein which is one of the low molecular weight MAPs, is of special interest. Normal tau, modulated by tightly regulated phosphorylation, stabilizes the α/β-heterodimers. However, in the idiopathic disease process of LOAD, tau is hyperphosphorylated and slowly forms intraneuronal inclusions and NFTs. Motor proteins use ATP hydrolysis to move cargo, including the MT proteins themselves, along this dynamic MT polymer (Hirokawa, Noda et al. 1998).

MT regulation may happen at many different stages, via transcription of different tubulin isotypes, the control of tubulin monomer folding, the formation of functional dimers, the posttranslational modification of tubulin subunits, the nucleation of MTs, or during the dynamic addition and disassociation of MT subunits in a dynamic polymer (Nogales, Wolf et al. 1998; Nogales 1999).

Approximately six functional genes encode α-tubulin isotypes in mammals (Field, Collins et al. 1984; Cleveland 1987; Luduena 1998). Alpha-III tubulin, encoded by b-alpha-1 gene, is highly expressed in brain. Blot analyses using RNA from a variety of transformed cells derived from different tissues indicate that expression of the human alpha-tubulin gene is restricted to cells of neurological origin (Hall and Cowan 1985). Among neurological cell types b-alpha-1 expression is further restricted to adherent cells that are morphologically differentiated. The data presented suggest that the b-alpha-1 gene encodes a prominent neuronal and glial alpha-tubulin and that b-alpha-1 expression is a

function of the differentiated state of these cells. Like, α-III tubulin, β-III tubulin has very high expression during development that deceases during post-natal and adult stages in mouse and rat (Miller, Naus et al. 1987; Miller, Naus et al. 1987). This α-III tubulin subtype is also increases in AD (Miller and Geddes 1990).

Alpha and β-tubulins shared 40% homology, and exhibit the greatest divergence at the C-terminus. The longest unbroken stretch of identical amino acids between all the α- and β-tubulins is found in positions 180-186 (Val-Val-Glu-Pro-Tyr-Asn), a region that is important for binding the guanosine tri-phosphate (GTP) (Little and Seehaus 1988) in both α and β isoforms. Seven α-tubulin isotypes have been identified in mammalian cells, encoded by different genes and with a distinct pattern of tissue expression (Luduena 1998).

Several studies have provided strong evidence for a functional role of the tissue-specific expression of individual β-tubulin isotypes (Joshi and Cleveland 1989; Raff, Fackenthal et al. 1997). Several of these isotypes, specifically class II β-tubulin (encoded by the Hβ9 gene), class III β-tubulin (encoded by the Hβ4 gene), and class IVa β-tubulin (encoded by the H5β gene), are highly expressed in cells of neuronal origin (Luduena 1998). The βIII isotype has been discovered as a taxol resistant tubulin in medulloblastomas and astrocytomas (Katsetos, Del Valle et al. 2001; Katsetos, Legido et al. 2003). From these studies and those conducted with chick embryogenesis, β-III tubulin is thought to be neuron-specific in brain (Lee, Tuttle et al. 1990).

Each MT is formed by the parallel association of protofilaments, linear polymers of tubulin dimers that are bound head to tail. The tubulin sequence and structure contain the information required for the self-assembly of protofilaments into polar, dynamic MTs, which in turn interact with a variety of cellular factors. This rapid polymerization/depolymerization cycle, termed treadmilling, is the net flow of MT subunits from the plus end that "grow" toward the axonal bouton or growth cones to the minus end that polarizes toward the MT organizing center at the cell body without a significant change in MT length (Nogales, Wolf et al. 1998; Nogales 1999). Treadmilling is essential to MT function. Alpha and β-tubulins are arranged in a strict head to tail fashion, such that the plus end is oriented away from the cell body and the minus end, toward the cell body within the axon. In dendrites, MTs form shorter protofilaments with a mixed orientation of plus and minus ends.

MTs are highly dynamic and can switch stochastically between growing and shrinking phases, both in vivo and in vitro. This non-equilibrium behavior, known as dynamic instability (Desai and Mitchison 1997), is based on the binding and hydrolysis of GTP by tubulin subunits. Each tubulin monomer binds one molecule of GTP. The binding to α-tubulin at the N site is non-exchangeable, whereas the binding to β-tubulin at the E site is exchangeable. Only dimers with GTP in their E site can polymerize, but, after polymerization, this nucleotide is hydrolyzed and becomes non-exchangeable, thus stabilizing the formation of the protofilament. The most favored hypothesis to explain dynamic instability is the GTP cap model (Mitchison and Kirschner 1984). In this model the body of the

MT, made of GDP-tubulin subunits, is unstable, and the MT structure is stabilized by a layer of tubulin subunits at the ends that still retain their GTP. When this cap is stochastically lost, the MT rapidly depolymerizes. MT assembly and stability, which are self-regulated by the nucleotide state of tubulin, are further modified in the cell by interaction with cellular factors that stabilize or destabilize MTs at different points in the cell or at different stages in the cell cycle (Hirokawa, Noda et al. 1998; McNally 1999) and during axonal growth and retraction.

The C-terminus of tubulins immediately follows helices H11 and H12 in the dimeric tubulin structure and is thought therefore to be located on the outer surface of the MT. The failure to resolve these residues in the crystal structure could be due to the fact that they are disordered, as indicated by some structural studies on synthetic carboxy-terminal peptides (Jimenez, Evangelio et al. 1999). The carboxy-terminal sequences of α- and β-tubulin are highly acidic and constitute the isotype defining regions (Sullivan and Cleveland 1986), where the various tubulin isotypes differ most strongly from each other .

The carboxy-terminal ten residues of α-tubulin and the carboxy-terminal 18 residues of β-tubulin (Nogales, Wolf et al. 1998) are the site of the heterogeneity of tubulin isoforms, binding site for MAPs, and the site of many post translational modifications including tyrosination/detyrosination of alpha tubulin, polyglycation which may include presence of AGEs found in AD brain tissue, polyglutamination, palmitoylation, and phosphorylation (Picklo, Montine et al. 2002; Westermann and Weber 2003). These modifications affect MAPs binding and MT dynamics. With the exception of AGEs, the aforementioned

post translational modifications occur under normal physiology. However, post translational modifications may take the form of molecules modified by oxidative damage in AD (Montine, Reich et al. 1998; Smith, Sayre et al. 1998; Conrad, Marshall et al. 2000; Head, Garzon-Rodriguez et al. 2001; Castegna, Aksenov et al. 2002; Castegna, Aksenov et al. 2002; Castegna, Aksenov et al. 2002; Choi, Malakowsky et al. 2002; Korolainen, Goldsteins et al. 2002; Castegna, Thongboonkerd et al. 2003).

1.7 Glial Activation and Oxidative Damage

Oxidative stress is defined as a disturbance in the prooxidant-antioxidant balance in favor of the former, leading to potential damage (Sies 1997; Roediger and Armati 2003). The central nervous system (CNS) seems to be especially vulnerable to oxidative stress on account of its high rate of oxygen utilization and the fact that neuronal membranes contain a high proportion of oxidation-prone polyunsaturated fatty acids (PUFAs), making them more susceptible to peroxidative damage (Sayre, Perry et al. 1999). Also, the brain appears to contain lower levels of molecular antioxidants such as superoxide dismutase and glutathione peroxidase (Floyd 1999; Sayre, Perry et al. 1999) as well as lower activities of antioxidant enzymes such as catalase (Halliwell 1992) and decrease in peptide methionine sulfoxide reductase in AD brain (Gabbita, Aksenov et al. 1999). Given the sensitivity of the brain to free radical damage, it is not surprising that oxidative stress has been implicated in a number of human degenerative disorders of the CNS (Smith, Richey Harris et al. 1997).

One of the major outcomes of oxidative stress in brain is lipid peroxidation. LPO products are generated from free radical attack on PUFAs like arachidonic acid (AA) and docosohexanoic acid (DHA) and their normally generated multiple products, including hydroxyl alkenals, neuroprostanes, and malondialdehyde among many others (Esterbauer, Schaur et al. 1991; Zollner, Schaur et al. 1991; Montine, Quinn et al. 2003) . LPO is increased in the central nervous system (CNS) in patients with AD both early and late in the course of their illness (Montine, Neely et al. 2002). Montine et. al. accomplished this by employing quantitative in vivo biomarkers of lipid peroxidation, F2-isoprostanes (IsoPs) and F4-neuroprostanes (NeuroPs) (Montine, Quinn et al. 2004). Importantly, LPO is different from other forms of oxidative damage because it is a self-sustaining process that will proceed until terminated by anti-oxidants or until substrate is consumed.

While LPO directly damages the biophysical properties of membranes, the major deleterious effect of LPO is believed to be through the generation of chemically reactive secondary products that modify cellular macromolecules, especially protein. One of the principal secondary products of LPO is 4-hydroxy-2-nonenal (HNE). We and others have shown that HNE-protein adducts accumulate in brains of patients with AD (Markesbery and Lovell 1998; Markesbery and Carney 1999), and are localized to neuronal cytoplasm, and others have suggested that HNE is a major effector of the neurotoxic effects of Aβ peptides.

How HNE and other reactive products of LPO may promote cytoskeletal abnormalities is not fully understood. HNE and related reactive products of LPO products exert their effects in tissue by reacting with lysine, cysteine, histidine, and arginine side chains of amino acids to form pyrrole and Michael adducts on proteins that lead to altered activity of enzymes and structural proteins (Sayre, Arora et al. 1993; Nadkarni and Sayre 1995; Cohn, Tsai et al. 1996; Montine, Kim et al. 1997; Montine, Reich et al. 1998). Many of these studies found that MT polymerization and structural organization was greatly impaired after direct application of LPO products or peroxynitrite (ONOO-) (Olivero, Miglietta et al. 1990; Miglietta, Olivero et al. 1991; Neely, Sidell et al. 1999; Landino, Hasan et al. 2002; Landino, Skreslet et al. 2004; Neely, Boutte et al. 2005).

Numerous studies of human tissue have employed antibodies to localize oxidatively modified proteins; broadly, these studies have localized oxidation, glycoxidation, nitrative, and LPO protein adducts to neuron cytosolic proteins and sometimes to NFTs in AD (Aksenov, Aksenova et al. 2000; Conrad, Marshall et al. 2000; Aksenov, Aksenova et al. 2001; Butterfield, Drake et al. 2001; Castegna, Aksenov et al. 2002; Korolainen, Goldsteins et al. 2002; Castegna, Thongboonkerd et al. 2003; Reynolds, Berry et al. 2005; Zhang, Xu et al. 2005). In addition to these patient-oriented studies, rodent models of AD also have been reported to show increased indices of oxidative damage, even before the deposition of Aβ-immunoreactive plaques (Butterfield 2002; Butterfield, Castegna et al. 2002; Butterfield and Castegna 2003).

Protein nitration is an increasingly recognized target of study as it is also part of the grand scheme of damaging oxidative chemistry. Reactive nitrogen species are initially generated by the upregulation of nitric oxide synthases. There are 4 isoforms differentially expressed with tissue specificity associated with brain and the blood-brain barrier: neuronal (nNOS), glial or inducible (iNOS), and endothelial (eNOS) (Lowenstein, Dinerman et al. 1994). NO is the product of nitric oxide synthase in an unusual reaction that converts arginine and oxygen into citrulline and NO. The mechanism of nitric oxide synthesis is not completely understood, but it involves the transfer of electrons between various cofactors, including flavin adenine dinucleotide, flavin mononucleotide, nicotinamide adenine dinucleotide phosphate, tetrahydrobiopterin, and heme within the mitochondrial oxidative phosphorylation pathway. Finally, one atom of oxygen from oxygen binds with the terminal guanidine nitrogen from arginine to form NO (McMillan, Bredt et al. 1992; White and Marletta 1992; Lowenstein, Dinerman et al. 1994).

NO, first identified as endothelium-derived relaxing factor, is produced by different types of cells in multicellular organisms where it acts as a diffusible messenger in many forms of intercellular communication as well as of intracellular signaling (Contestabile, Monti et al. 2003; Contestabile and Ciani 2004) (Moncada, Palmer et al. 1989; Bredt and Snyder 1992). Its role is as a brain messenger molecule, acting as an unconventional neurotransmitter or neuromodulator. Neuronal NOS is hypothesized to be expressed when the supply of target-derived trophic factors is absent during early embryogenesis or

during injury and, therefore, acts as a neurotrophic signaling molecule. Neuronal NOS expression actually increases in cultured dorsal root ganglion (DRG) neurons that are “starved” of nerve growth factor (NGF) (Thippeswamy and Morris 1997; Thippeswamy, Jain et al. 2001). It was, therefore, supposed that NO could be able to protect neurons whose survival was endangered by NGF deprivation. Furthermore, neurons cultured from nNOS knockout mice, are more vulnerable to death than in wild-type animals (Keilhoff, Fansa et al. 2002).

Unlike neuroprotective processes involved in neural differentiation, the imbalance or uncontrolled NOS production proceeds unchecked in neurodegenerative diseases wherein oxidative modification of a compromised neuron as opposed to a newly generated cell leads to disastrous consequences. Inflammation in the brain primarily involves the participation of the two types of glial cells, microglia and astrocytes (Kreutzberg 1996; Aloisi 1999). Under physiologic conditions; microglia, the resident immune cells in the brain; serve a role of immune surveillance. Astrocytes, on the other hand, principally maintain ionic homeostasis, buffer the action of neurotransmitters, and secrete nerve growth factors. However glia, especially microglia, readily becomes activated in response to immunologic challenge and injury (Aloisi 1999). Activation of glia, a process termed reactive gliosis, has been observed during the pathogenesis of Parkinson’s disease, AD, multiple sclerosis, and AIDS dementia complex, as well as post-neuronal death in cerebral stroke and traumatic brain injury (Dickson, Lee et al. 1993; O'Banion and Finch 1996; Hauss-Wegrzyniak, Dobrzanski et al. 1998). In response to LPS stimulation, used in many cell culture and mouse

models of inflammation, microglial production of NO and other reactive oxygen and nitrogen species leads to neuronal death (Shie, Milatovic et al. in press).

Cells can greatly increase the toxicity of other ROS from the reaction of superoxide and NO to produce ONOO–, one of the fastest reactions known in biology (Beckman 1994). In the presence of carbon dioxide, ONOO- readily modifies proteins to form nitrotyrosine. Nitrotyrosine can be also formed by peroxidase oxidation of nitrite, a byproduct of NO metabolism, and hydrogen peroxide (Brennan, Wu et al. 2002). NO can react with membrane lipids to induce lipid peroxidation. Indirectly, the combination of NO and superoxide ($O_2^{\bullet -}$) can form highly reactive intermediates, such as ONOO-, that can induce DNA strand breaks, lipid peroxidation, and protein nitration (Beckman 1996).

The significance of NO contribution to neuronal injury is indicated by the use of nitric oxide synthase (NOS) inhibitors and is best documented by the use of mutant mice deficient in the neuronal isoform of NOS (nNOS) (Hantraye, Brouillet et al. 1996; Przedborski, Jackson-Lewis et al. 1996; Ayata, Ayata et al. 1997; Eliasson, Huang et al. 1999; Liberatore, Jackson-Lewis et al. 1999). Older mice deficient in nNOS were found to be resistant to related neurodegenerative diseases, such as stroke, N-methyl-D-aspartate neurotoxicity (NMDA), and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity in models of Parkinson's disease (Przedborski, Kostic et al. 1992). In addition to nNOS, other studies in human and animal models have also documented the contribution of iNOS, the inducible form of NOS, primarily found in brain glial cells (Vodovotz, Lucia et al. 1996; Liberatore, Jackson-Lewis et al. 1999) . For example, plaques

in multiple sclerosis patients showed increased immunoreactivity for iNOS and nitrotyrosine (Bagasra, Michaels et al. 1995). Nitration has also been associated with compromised integrity of the blood-brain barrier in multiple sclerosis (Kean, Spitsin et al. 2000). NO derived from iNOS also contributes to neurotoxicity in ALS mouse models as well as in the MPTP model of Parkinson's disease. Blockade of iNOS and microglia activation has been found to be neuroprotective in neurodegenerative diseases mouse models, (Wu, Jackson-Lewis et al. 2002; Zhu, Stavrovskaya et al. 2002). Activation of microglia leading to ONOO- formation has also been linked to Aβ peptide neurotoxicity (Xie, Wei et al. 2002; Ischiropoulos and Beckman 2003).

NO, like many other molecules of oxidative damage, also modifies MT protein directly. Nitro-tyrosine is transported into mammalian cells and selectively incorporated into the extreme carboxyl terminus of α-tubulin, changing MT organization and interaction with motor proteins (Eiserich, Estevez et al. 1999). Donors of NO lead to a high degree of axonal retraction in cultured chick sensory neurons (He, Yu et al. 2002). Nitration also occurs on the MAP tau in differentiated PC12 cells (Cappelletti, Tedeschi et al. 2004). Recently, nitration has been observed using mass spectrometry methods in AD, directly associating oxidative damage to AD pathology (Castegna, Thongboonkerd et al. 2003; Shin, Lee et al. 2004).

While the sources of oxidative damage and a true causal role in AD are not entirely resolved, they include the potentially partially overlapping processes

of Aβ peptide formation, tau aggregation, innate immune activation, mitochondrial dysfunction, and MT dysfunction.

1.8 Mass Spectrometry in Discovery of Protein Modification by Oxidative Damage

An increasing number of studies are using multiple methods of mass spectrometry to identify and quantitate oxidative species and oxidatively modified proteins in AD (Liu, Yeo et al. 1997; Castegna, Aksenov et al. 2002; Castegna, Aksenov et al. 2002; Castegna, Aksenov et al. 2002; Castegna, Thongboonkerd et al. 2003; Pamplona, Dalfo et al. 2005; Williams, Lynn et al. 2005) and in mouse models (Shin, Lee et al. 2004). An elegant series of experiments have coupled 2-dimensional gel electrophoresis (LC-LC) and immunochemical detection of proteins with carbonyl modifications with matrix assisted laser desorption ionization (MALDI) coupled to tandem time of flight mass spectrometry (TOF-TOF-MS) to obtain a broad view of proteins labile to this form of oxidative modification in AD patients and in rats following intracerebral injection of $A\beta_{42}$ (Boyd-Kimball, Sultana et al. 2005). Another recent study determined the amount of 5 different amino acid modifications in frontal cortex from AD patients by quantifying modified amino acids by gas chromatography (GC)/MS (Pamplona, Dalfo et al. 2005). While these approaches have provided insight into the pathogenesis of protein modification in AD, both studies examined proteins extracted into aqueous buffer. Indeed, no study we are aware of has yet sought to associate oxidative protein modifications in AD with transition to detergent-insolubility, examined protein modification from the

perspective of amino acid sequence, or concentrated on neuronal cytoskeletal proteins, proposed to the site of earliest dysfunction in AD pathogenesis.

A scoring algorithm for spectral analysis (SALSA), which performs automated pattern recognition for peptide modifications in tandem mass spectral data, has been used to identify oxidative protein modifications in vitro (Badghisi and Liebler 2002; Liebler, Hansen et al. 2002), including $A\beta_{40}/Cu^{2+}$-induced histidine and methionine oxidation (Schiewe, Margol et al. 2004). P-MOD, similar to SALSA, is an algorithm and software that identifies and maps modifications to peptide sequences using tandem mass spectral data and includes calculation of error rates or p-values of peptide modifications (Hansen, Davey et al. 2005). This study combines well-established extraction methods with P-MOD analysis to identify and map oxidative modifications to four cytoskeletal proteins that are associated with the biochemical abnormality of detergent insolubility or accumulation into NFTs.

1.9 Summary

In the present studies, I have tested the hypotheses that reactive products of LPO are the effectors that lead to neuronal MT collapse and inability to polymerize in cells. This same loss of MT function is recapitulated in human brain tissue from LOAD, FTDP-17, but not MCI or in age-matched controls. Using mass spectrometry to define and map modifications, α-tubulin is modified by nitration, β-tubulin is extensively modified by oxidation, and tau is modified by LPO products, HHE and HNE, within functionally relevant protein regions. In this

study, MT dysfunction in human brain tissue parallels the increasing abundance of modifications induced by oxidative damage of MT proteins that is also in accordance with protein insolubility. Our proposed scheme for how these different facets of AD pathogenesis may be related is presented in the flowchart below, adapted from Hardy and Selkoe (Selkoe 2000).

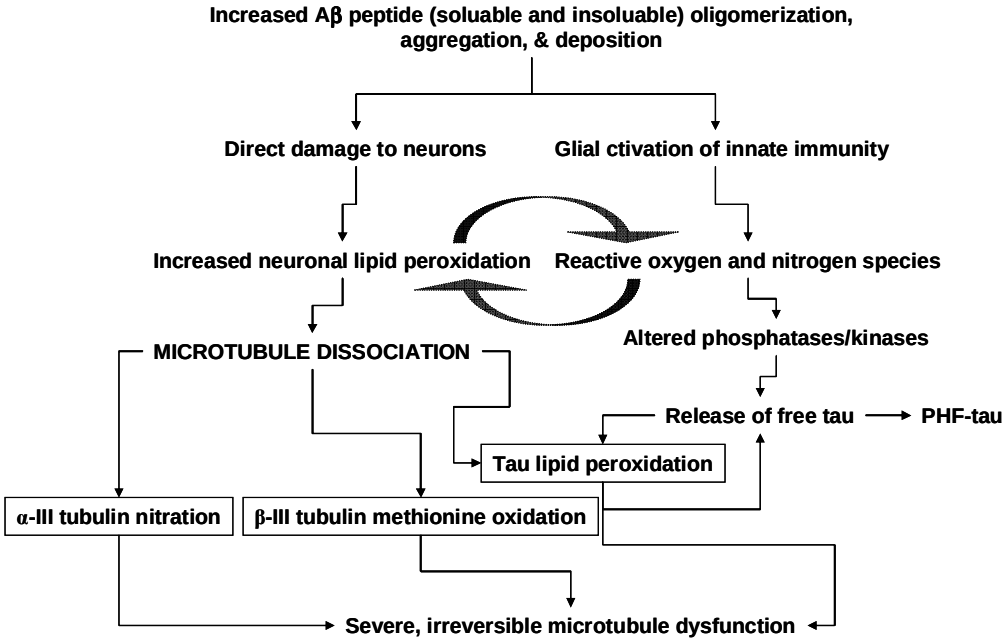

Figure 2. Modified model of AD pathogenesis adapted from Hardy and Selkoe, focusing on neuronal microtubule proteins.

1.10 Rationale and Specific Aims

The experiments described herein test the hypothesis that *neuronal cytoskeletal protein dysfunction found in AD is similar to that inflicted by LPO products and that oxidative damage is linked to pathological cytoskeletal protein aggregation*. Several factors underlie this hypothesis. Oxidative damage is a

well established early and on-going event in AD and may directly affect cellular proteins. Exposure of N2a cells to 10-25 μM HNE cells causes inhibition of neurite outgrowth and MT disruption at sub-cytotoxic concentrations through Michael addition chemistry (Neely, Sidell et al. 1999). This effect from HNE is due to its reaction with intracellular proteins, and the major cytosolic protein bound to HNE has a molecular weight of approximately 55 kDa, corresponding to neuronal tubulin and tau among other proteins (Keller, Mark et al. 1997). One limitation of previous studies is that they did not include a myriad of oxidative or other pathological events nor did they include a test of MT function and discreet assessment of protein post-translational modifications.

The architecture and function of MTs was further expanded to test the effects of HNE and related aldehydes, of a free radical generator, of glial mediated oxidative damage, and of increased intraneuronal accumulation of APP derived peptides. In addition, MT function in human tissue from patients with AD and related neurodegenerative diseases was investigated. While oxidative damage and aberrant protein structure and function have been linked, no study had yet shown changes or trends in oxidative modification of MT proteins at the amino acid level using mass spectrometry. To test the hypothesis that *neuronal cytoskeletal protein dysfunction found in AD is similar to that inflicted by LPO products and that oxidative damage is linked to pathological cytoskeletal protein aggregation*, the specific aims are as follows:

1. To determine if LPO products, free radicals, activated glia, or APP peptide products inhibits the functionality of MT.

2. To determine if MT function is similarly inhibited in neurodegenerative diseases.
3. To examine the type and location of oxidative modifications of cytoskeletal proteins that may explain or enhance the reason for loss of MT function and increased protein insolubility.

The goal of these studies is to further our understanding of the links between oxidative damage, protein modification, and protein aggregation that contribute to AD pathogenesis.

CHAPTER II

MECHANISMS OF NEURONAL MICROTUBULE DYSFUNCTION INDUCED BY MULTIPLE MODELS OF OXIDATIVE DAMAGE

2.1 Introduction

We have previously shown that LPO products lead specifically and potently to dissolution of neuronal MTs and decreased neurite outgrowth *in vitro* (Neely, Sidell et al. 1999). Indeed, MT collapse from exposure to HNE is the most sensitive endpoint yet studied for HNE in neuronal cells. Specifically, incubation of neuronal cells with 25 μM HNE for 15 minutes led to complete MT collapse. HNE is a major aldehyde product of LPO in brain; other LPOs also are produced in at least similar quantities, including acrolein and HHE. Therefore, experiments investigated the effects of these aldehydes and their effect on taxol-stimulated MT polymerization. Additional cell culture experiments tested the ability of glial mediated oxidative damage, of a free radical generator, and of endogenous amyloid peptide to lead to changes in tubulin morphology.

These data suggest that direct application of LPO products, but not indirect application via activated glial production of ROS, are the stimuli for MT collapse that is characteristic of AD. In contrast, increased amyloid peptide expression and aggregation induced an increase in tubulin self-association. Although, both Aβ peptides and PHF-tau appear to play central roles in AD, the link between them or common mechanisms behind their shared property of

aggregation into insoluble material and the degeneration of the cytoskeleton remains poorly defined. Mechanisms of HNE-induced neuronal MT dysfunction may have a critical role in MT function and morphology in cells, while Aβ and related peptides, like C99, may cause cytoskeletal dysfunction in another manner.

2.2 Materials and Methods

Materials

Chemicals required for the synthesis of 4-hydroxy-2(E)- nonenal (HNE), 4-hydroxy-2(E)- hexenal (HHE) , 1,1,4-Tris(acetyloxy)-2(E)-nonenal (HNE[Ac]3), and acrolein were purchased from Aldrich (Milwaukee, WI). HHE and HNE were synthesized as described (Amarnath, Valentine et al. 1998) (Gardner, Bartelt et al. 1992) and stock solutions in either dimethyl sulfoxide or ethanol were kept no longer than one week at 20 °C. The synthesis of 1,1,4- Tris(acetyloxy)-2(E)-nonenal (HNE[Ac]3) was performed as described (Neely, Amarnath et al. 2002) and acrolein was distilled prior to use. Unless otherwise indicated, materials used for cell culture were from Invitrogen (Grand Island, NY) and all other chemicals were from Sigma (St. Louis, MO).

Cell culture

Neuro-2a (N2a) neuroblastoma cells were purchased from American Type Culture Collection (Rockville, MD). For propagation, the cells were seeded at 20 $X10^4$ cells/ml in growth medium (Dulbecco's Modified Eagle Medium: Nutrient

Mixture F-12 (1:1) (DMEM/F12) containing 10% fetal bovine serum and penicillin–streptomycin at 100 units/ ml and 100 μg/ml, respectively, and subcultured twice weekly. Unless indicated otherwise, before all experiments, the cells were subcultured at the desired cell density and incubated overnight in growth medium. The next morning, the cells were washed with DMEM/F12 three times and incubated in serum-free N2-medium (DMEM/F12 containing penicillin/streptomycin and N2 supplement) for 24 h prior to aldehyde or (2-amidino- propane) dihydrochloride (AAPH) exposure for either fractionation or polymerization assays.

Glial Activation and Dual Well Plating

N2a cells were plated for microscopy as described (Neely, Sidell et al. 1999). Primary mouse microglia cultures were plated in an upper chamber of permeable membrane exactly as described (Shie, Breyer et al. 2005) and treated with 10μg/mL LPS (or Aβ 5μM to 0.31μM) for 36hrs.

Immunoflourescence

Alpha tubulin immunostaining for N2a cells used in the dual well plating system was performed as described (Neely, Sidell et al. 1999).

MC65 cells were plated at 2 X 10^4 cells/mL X 2mL per well on glass slides, previously incubated with 0.05mg/mL poly-D-lysine for 24 hrs at 37°C, in the presence of 10% FBS, 1% pen/strep in DMEM/ F-12 medium supplemented with 1.5ug/uL tetracycline to suppress C99 expression. After 24hrs to allow

adherence, cells were washed then incubated in 1X Optimem with tetracycline for 24hrs. The next day media was replaced with Optimem without tetracycline for 24, 48 and 72 hrs. Cells were washed with warmed 1XHBSS, fixed in 4% PF, then washed with 1X PBS. Cells were permeabilized and blocked with 0.5% triton and 2% FBS in 1X PBS 10 minutes, then incubated with 1:4000 αtubulin antibody at 4°C for 3hr at RT. For detection, cells were incubated at 1:100 FITC conjugated and anti-mouse IgG antibody for 2 hrs.

Tubulin Fractionation

Cells plated as described and incubated in 1% N2 media with either AAPH or HNE. Cells were harvested into pipes extraction buffer (80mM PIPES, 2mM EGTA, 30% glycerol) with protease inhibitors and 0.1% triton, and centrifuged at 14, 000 rpm on a table top centrifuge. Supernatant was boiled for 10min and pellets resuspended in an equal volume of 2XSDS "stop" buffer (500mM Tris, pH 6.8, 10%SDS, 100mM EGTA, 100mM EDTA, 10% glycerol).

Tubulin Polymerization

13.5 ml of N2a cells at 20 X 10^4 cells/ml were seeded in T-75 mm^2 flasks and prepared for the experiment as described in the Cell culture section. Cells were exposed to varying concentrations of either HNE or HNE(Ac)3, harvested with a cell scraper and sedimented. Cell pellets were washed two times with Hank's Balanced Salt Solution (Invitrogen, Grand Island, NY) and then

reconstituted in 200 μl of cold extraction buffer (100 mM 2-[N-morpholino] ethanesulfonic acid (MES), 1 mM ethylenglycol-bis(2- aminoethylether)-N,N,NW,NV-tetraacetic acid (EGTA), 0.5 mM MgCl2, 4 M glycerol, 2 mM guanosine 5V-triphosphate (GTP), protease inhibitor cocktail diluted 1/10,000 (Sigma, St. Louis, MO, #P 8340). Samples were then pulse sonicated (2 times for 20 s at 20 W) and the cell lysates centrifuged (100,000 g, 4 8C, 1 h). The pellet (P1) was reconstituted in a volume of extraction buffer equal to the volume of the sample cell lysate. The protein concentration of the supernatant (S1) was determined using the Dc Protein assay kit according to the manufacturers instructions (BioRad, Hercules, CA). Protein concentrations in S1 samples were then adjusted to be 1.2 mg/mL. S1 solutions with protein concentrations below 1.2 mg/mL were concentrated with Millipore Ultrafree centrifugal concentrators (Fisher Scientific, # UFV5BGC25, Swanee, GA). Taxol and GTP were added to final concentrations of 40 μM and 2 mM, respectively, the samples incubated in a shaking water bath for 30 min at 37°C and then centrifuged (100,000 g, 37°C, 1 h). The supernatants (S2) were removed and the pellets (P2, polymerized tubulin) homogenized in a volume of extraction buffer equal to S1. All samples were stored at 80 °C until further analysis by immunoblot analysis. Tubulin polymerization of MC65 cells was performed as above. 24h after initial plating in 10%FBS containing media, cells were incubated in 1XOpti-MEM for another 24h, both in the presence of tetracycline. The next day, media was replaced without tetracycline for 24h.

Immunoblot analysis

Samples of the tubulin polymerization assay were diluted in Laemmli sample buffer, proteins separated by SDS-PAGE (Laemmli 1970) and transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA). For visualization of tubulin, the membrane was first incubated with blotto(4% dry milk in Tris-buffered saline with 0.2% Tween 20) to block unspecific antibody binding sites, then with anti-α-tubulin (Sigma, St. Louis, MO, #T5168) diluted 1000-fold in blotto, followed by HRP-conjugated anti-mouse IgG (Amersham, Piscataway, NJ, #NA931) diluted 2000-fold in blotto. All incubations were for at least 2 h at room temperature or overnight at 4 °C. The signal was developed using a chemilluminescence reagent (PerkinElmer Life Sciences, Boston, MA, # NEL103) and visualized on Kodak X-OMAT AR film (Sigma, St. Louis, MO).

2.3 Results

We have previously shown that exposure of N2a cells to HNE results in the adduction of this aldehyde to tubulin, but the functional significance of this modification had not been demonstrated (Neely, Sidell et al. 1999; Neely, Zimmerman et al. 2000). Here, we studied the extent of taxol-induced tubulin polymerization in control and HNE-treated N2a cells. Taxol is a diterpene derived from the yew tree Taxus brevolia, and allows tubulin polymerization to proceed independent of MAPs (Schiff and Horwitz 1981). Exposure of N2a cells to HNE resulted in a concentration and time-dependent inhibition of taxol induced tubulin polymerization (Figs. 3A,B). Taxol-induced tubulin polymerization was inhibited in

N2a cells exposed to HNE concentrations of 10 μM (Figure 3A) and after exposure times as short as 15 min (the shortest time we tested) (Figure 3B). HNE(Ac)3, the intracellularly activated analogue of HNE, inhibited taxol-induced tubulin polymerization to a similar degree as HNE (Figure 3C).

Compared to HNE, HNE(Ac)3 compound was about two times less efficacious. HNE(Ac)3 has previously been shown to be about two times less efficacious in MT disruption than HNE (Neely, Amarnath et al. 2002) . The difference in reactivity is likely due to the fact that HNE(Ac)3 is partially hydrolyzed into HNE(Ac)1 (Neely, Amarnath et al. 2002). HNE(Ac)1, just like HNE, is an α-β-unsaturated aldehyde and therefore a strong electrophile. However, HNE(Ac)1 has an acetyloxy group on the 4 position. This acetyloxy group is expected to confer more steric hindrance than the hydroxyl group present at that same position in HNE.

An alternative method to assessing altered function of tubulin is to isolate assembled, membrane bound tubulin from free tubulin directly from cells. Disruption of MTs leads to a shift in the distribution of tubulin with less assembled as MTs and more free tubulin in cytosol. Figure 4 presents data from N2a cells exposed to increasing concentrations of HNE for 1 hr. These results gave a similar conclusion compared to the immunoflourescence microscopy and *in vitro* polymerization assay, viz, approximately 10 μM HNE is the EC_{50} for a dramatic increase in the amount of free (S) tubulin and a decrease organelle bound, assembled (P) tubulin in N2a cells.

Figure 3.

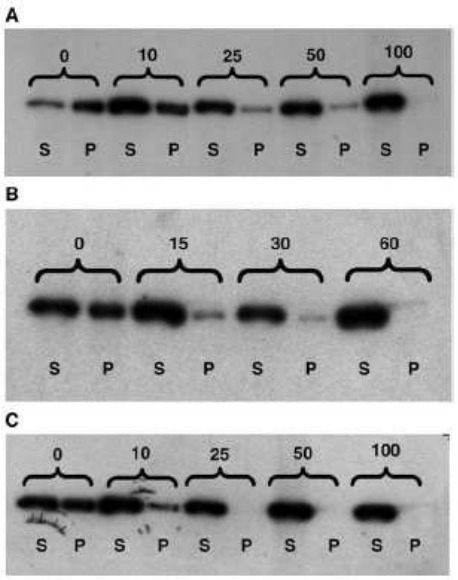

Figure 3 (A) HNE causes concentration-dependent inhibition of taxol induced tubulin polymerization. After N2a cells were exposed to a range of HNE concentrations (0–100 μM) for 1 h, tubulin polymerization was induced in the cytosolic fraction with taxol. Microtubules were sedimented by ultracentrifugation and the tubulin content in the pellet (P, microtubules) and the supernatant (S, unpolymerized tubulin) compared by immunoblot analysis using anti-tubulin antibodies. While in control cells, the majority of tubulin is found in the pellet, the fraction of tubulin in the microtubule pellet decreases as the HNE-concentration increases. (B) HNE induced inhibition of taxol-induced tubulin polymerization is time dependent. Taxol-induced polymerization of cytosolic tubulin of N2a cells exposed to 25 μM HNE for varying times was analyzed. The effect of HNE is rapid, such that within 15 min of exposure, the majority of tubulin is observed in the supernatant(S), while in control cells, the majority of tubulin is isolated in the microtubule pellet (P). (C) HNE(Ac)3 inhibits cytosolic taxol-induced tubulin polymerization. N2a cells were exposed to a range of concentrations of HNE(Ac)3 for 1 h and the taxol-induced polymerization of cytosolic tubulin was analyzed. Similar to HNE, HNE(Ac)3 caused substantial inhibition of taxol-induced tubulin polymerization at 10 □M (S, unpolymerized tubulin; P, microtubule pellet). Immunoblots in these figures are representative of a total of three experiments for each variable.

Figure 4.

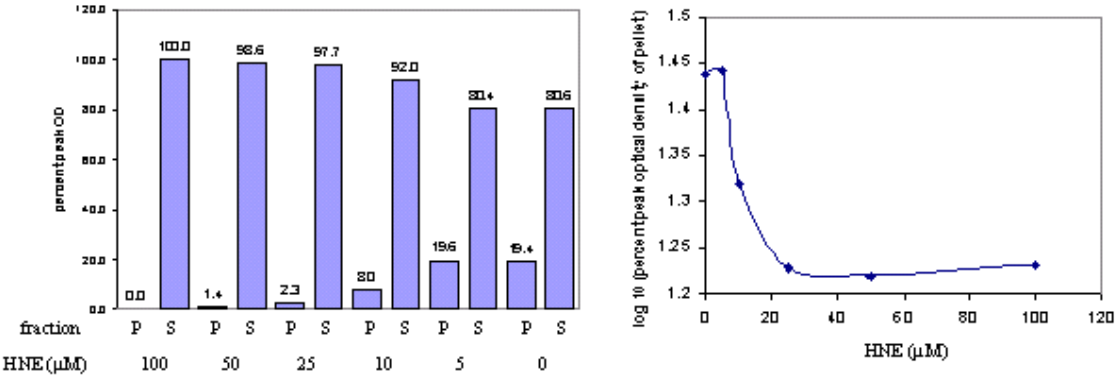

Figure 4. Percent of tubulin from N2a cells assembled in pellet (P) and free in supernatant (S) following exposure to HNE. (A) Percent distribution of tubulin in P and S fractions at different HNE concentrations (μM). (B) Percent tubulin in P fraction expressed as $\log_{10}$ at different HNE concentrations. N2a cells (ATCC #CCL-131) were exposed to HNE for 1 hour. 25μM or greater HNE treatment increases percent of free tubulin (S); conversely, percent of bound tubulin (P) is significantly diminished. Western blots were performed as in figure 1. Peak optical density was determined by using Quantity One (Biorad) and expressed as % of tubulin in P or S for each sample.

Previously we have observed that N2a cells exposed to HNE are deficient in taxol-stimulated (MAP independent) polymerization of tubulin extracted from cells. Although HNE is a major aldehyde product of LPO in brain, others reactive aldehydes, LPOs, are also produced in at least similar concentrations. These include acrolein and HHE. Therefore, we extended our previous experiments to include investigation of these aldehydes and their effect on taxol-stimulated MT polymerization. An example of our data for α tubulin is presented in Figure 5 that shows reduced polymerization of extracted tubulin following incubation of cells with HHE (5A) or acrolein (5B). These data demonstrated the capacity of different aldehydes from LPO to irreversibly alter the ability of α-and β-tubulin to polymerize independently from MAPs (Figure B). Representative Western blot of α-tubulin from acrolein-exposed N2a cells. N2a cells were exposed to 5-100

μM acrolein for 1hr, solubilized, and the extract subjected to GTP/taxol-induced tubulin polymerization. P2 and S2 are as described in Figure 3. MT polymerization was abolished by exposure to 25 μM and higher.

Results from our concentration-response experiments are presented in Figure 6; these data are for α-tubulin although virtually identical results were obtained when the same analysis was performed for β-tubulin (not shown). All three aldehydes progressively suppressed the capacity of taxol-stimulated tubulin polymerization. While the maximal effect of all three aldehydes was similar, their EC_{50}s were significantly different. The EC_{50} (± 95% confidence interval) for acrolein was 23 ± 4 μM, for HNE was 46 ± 9 μM, and for HHE was 75 ± 12 μM (ANOVA had $P < 0.01$).

Figure 5.

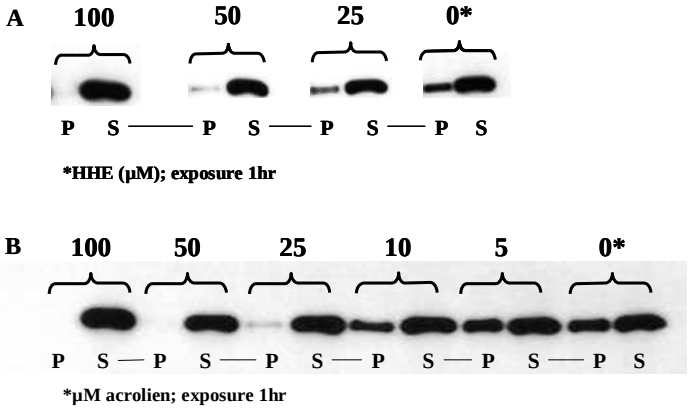

Figure 5. Representative Western blot of α-tubulin from HHE (A) and acrolein-exposed (B) N2a cells. N2a cells were exposed to 5-100 μM HHE or acrolein for 1hr, solubilized, and the extract subjected to GTP/taxol-induced tubulin polymerization as previously described.

Figure 6.

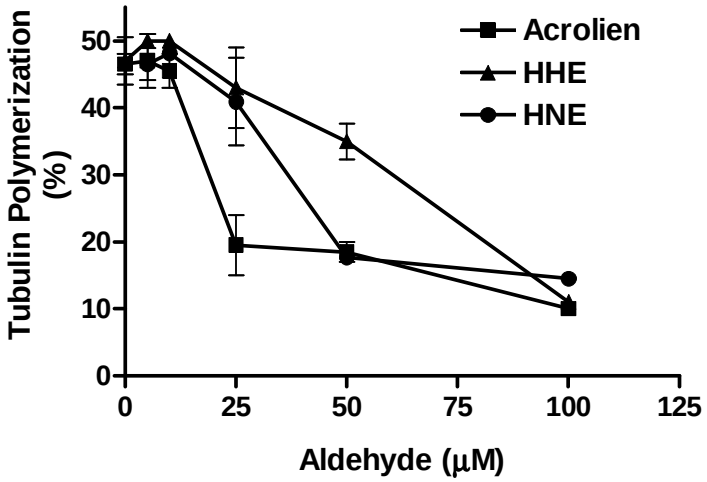

Figure 6. Concentration response relationships for taxol-stimulated tubulin polymerization following aldehyde incubated with N2a cells. Data are % of α-tubulin polymerized relative to total α-tubulin extracted from cells + SEM (n > 4 per data point). The calculated EC50s were 23 + 4 μM, for HNE was 46 + 9 μM, and for HHE was 75 + 12 μM (ANOVA had $P < 0.01$).

MC65 cells produce C99, a cleavage product of APP after cleavage by γ-secretase (Jin, Hua et al. 2002). In addition, C99 expression is followed by an intracellular increase of isoPs (Woltjer, Nghiem et al. 2005). Expression is under control of the tetracycline promoter; therefore, tetracycline removal allows C99 expression. In this study, under conditions similar to those previously described, immunoflourescence for α-tubulin showed increased aggregation of tubulin about the cell body and retraction away from the cells neurites after 24 hours of C99 expression. This change in cytoskeletal morphology was most drastic after 48 hours of C99 expression (Figure 7). No neurites were apparent and nearly all tubulin was localized to the cell body. At both 24 and 48 hours in the absence on C99 production, cells maintained normal, unchanged tubulin morphology.

Figure 7.

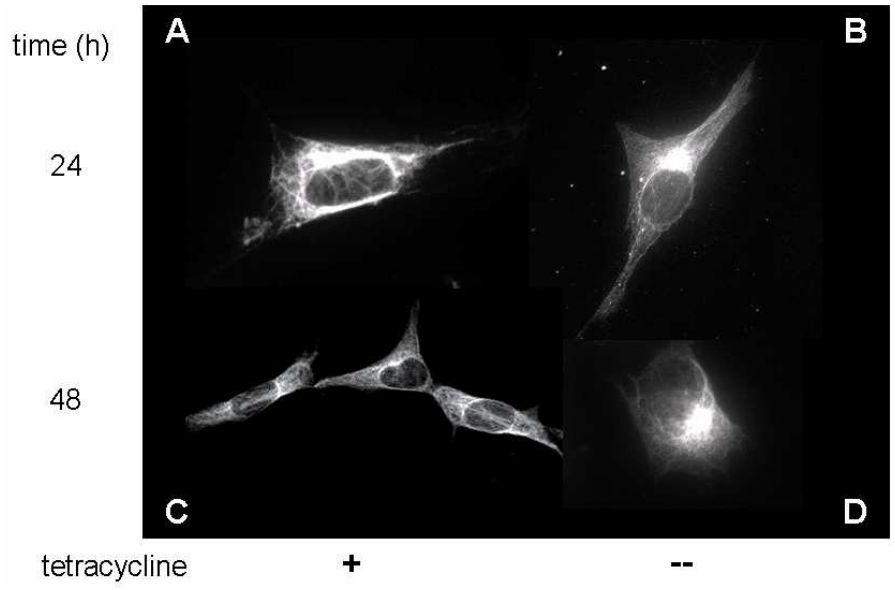

Figure 7. Amyloid precursor protein cleavage product, C99, expression alters microtubule morphology in MC65 cells. MC65 cells were incubated on glass slides in the presence (+) or absence (-) of tetracycline and stained for α-tubulin. C99 expression (tetracycline (-)) leads to increased localization of α-tubulin near the cell body after 24h (B). Neurites are completely abolished after 72h (D). In the absence of C99 (tetracycline (+)), α-tubulin morphology remained unchanged at 24h (A) and 72h (C).

MC65 cells were also assayed for tubulin polymerization ability using our taxol-GTP method (Figure 8). Contrary to the effect of free reactive aldehydes, tubulin polymerization increased with increasing levels of C99 expression. After 24 hours of C99 expression, tubulin polymerization was nearly unchanged compared to cells not expressing C99. After 48 and 72 hours tubulin polymerization was nearly 60% and 150% greater.

Figure 8.

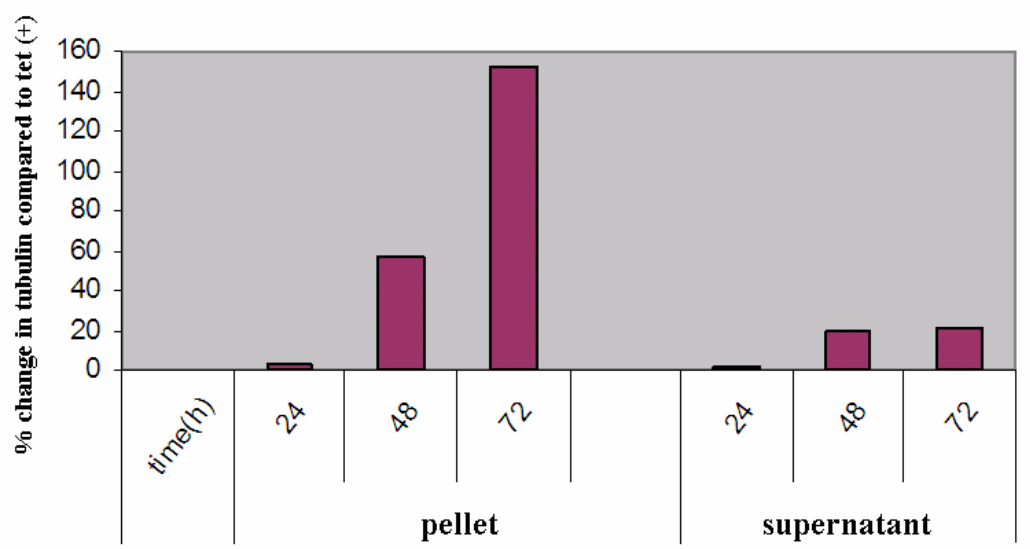

Figure 8. C99 expression increases tubulin polymerization. Taxol/GTP tubulin polymerization was tested using MC65 cells and immunoblots were probed with α-tubulin. Values are expressed as the percent (%) change in α-tubulin in C99 expressing cells compared to non-expressing cells. Tubulin polymerization increased by 60% after 48h and by 160% after 72h C99 expression. Unpolymerized tubulin in the supernatant increased to 20% after 48h of C99 induction, but was unchanged after 72h.

Tubulin fractionation is an assay of tubulin dynamics or a comparative measure of assembled (P) vs. non assembled (S) tubulin. N2a cells treated with AAPH, a compound that generates free radicals in solution, showed no change in fractionation (not shown). However, HNE treated cells had a drastic decrease in tubulin in the pellet fraction as stated above.

Lastly, using activated microglia, N2a cells were tested for MT integrity. Although, the concentrations of Aβ and LPS used lead to abundant microglial activation and neuronal cell death (Xie, Smith et al. 2004; Feng-Shiun Shie 2005;

Shie, Breyer et al. 2005) , these conditions failed to induce cytoskeletal changes assayed by α-tubulin microscopy (not shown).

2.4 Discussion

HNE also adducts to cellular proteins (Dickinson, Iles et al. 2002). In vitro incubation of purified bovine brain MT protein (tubulin and MAPs) with HNE results in a reduction in the rate and extent of tubulin polymerization (Gabriel, Miglietta et al. 1985; Olivero, Miglietta et al. 1990; Miglietta, Olivero et al. 1991). However, extrapolations of such in vitro observations with purified proteins to cellular events are difficult. The polymerization of purified tubulin depends strongly on type of buffers used, divalent cations, MAPs added, and other factors (Gillespie 1975; Banerjee, Jordan et al. 1985; Bayley, Schilstra et al. 1989). The cellular (in vivo) regulation of MT dynamics is very complex and involves MAPs, other cytoskeletal elements, and signaling cascades (Howard and Hyman 2003). In addition, issues such as membrane permeability of extracellularly applied HNE and nature of actual cellular target proteins have to be addressed. In previous experiments, we demonstrated that tubulin is a major target of extracellularly applied HNE (Neely, Sidell et al. 1999; Neely, Zimmerman et al. 2000). However, the functional significance of this tubulin modification had not been examined. Here, we developed an assay to study the effects of HNE on the functionality of tubulin of live cells that had been exposed to HNE. Specifically, we used taxol to induce tubulin polymerization in the cytosolic fraction of N2a cells that had been exposed to HNE or HNE(Ac)3. Taxol induces tubulin

polymerization independent of MT regulatory proteins, such as the MAPs (Schiff and Horwitz 1981) . Therefore, the characteristics of taxol-induced tubulin polymerization in a cytosolic fraction reflects the functionality of the tubulin protein itself. We observed that taxol-induced tubulin polymerization is inhibited in the cytosol of N2a cells that had been exposed to HNE. The time and concentration dependence of the HNE-induced inhibition of taxol-induced tubulin polymerization correlate well with our previous immunocytochemical observations (Neely, Sidell et al. 1999).

These observations, together with our finding that tubulin is a main cellular target protein of HNE (Neely, Sidell et al. 1999; Neely, Zimmerman et al. 2000) , support the notion that HNE-adduction to tubulin is the primary cause of the HNE-induced loss of cytoplasmic MTs, although we cannot exclude the possibility that a possible modification of MT regulatory protein plays an additional minor role. Landino and collaborators found the in vitro peroxynitrite-induced oxidation of tubulin sulfhydryls to be the primary reason for the inhibition of tubulin polymerization, whereas the oxidation of SH groups on MAPs played a minor role (Landino, Hasan et al. 2002; Landino, Skreslet et al. 2004) .

To our knowledge, this is the first demonstration of the mechanism by which HNE disrupts cellular MTs in living cells, namely, by adducting to tubulin and thereby inhibiting its polymerization. In addition, we demonstrated that exposure of neuronal cells to pathophysiologically relevant concentrations of aldehydes derived from LPO resulted in irreversible reduction in taxol/GTP-stimulated tubulin polymerization and this effect was greatest for acrolein.

The MC65 cell line expresses a specific C-terminal fragment of APP, C99, that is generated by cleavage of APP by γ-secretase. MC65 cells are also hypothesized to produce Aβ fragments as assayed by SDS-PAGE migration. Both C99 and these smaller fragments accumulate and aggregate over time and are associated with increasing toxicity, mitochondrial dysfunction, and cell death (Jin, Hua et al. 2002; Woltjer, Maezawa et al. 2003; Maezawa, Jin et al. 2004; Woltjer, Nghiem et al. 2005). This increase in tubulin polymerization was not initially expected because Aβ is also a generator of oxidative damage (Butterfield and Kanski 2002) and C99 production leads to increased isoPs (Woltjer, Nghiem et al. 2005). Therefore, related fragments such as C99 were expected to be followed by MT collapse, which for our purposes is in part demonstrated by loss in polymerization. However, very recent studies indicate interaction between Aβ and tubulin (Verdier, Huszar et al. 2005). Based on this increase in tubulin within the polymerized fraction, C99 may be recruiting tubulin into amphorous aggregates. Tubulin has been shown to associate with Aβ peptides or APP C-terminal fragments (Baumann, Wisniewski et al. 1996; Islam and Levy 1997; Verdier, Huszar et al. 2005).

CHAPTER III

DIMINISHED BRAIN TUBULIN POLYMERIZATION FROM PATIENTS WITH DEMENTIA, BUT NOT INDIVIDUALS WITH MILD COGNITIVE IMPAIRMENT

3.1 Introduction

The neuronal cytoskeleton has long been proposed as a likely source of vulnerability to a variety of stressors because of the need for neurons to transport organelles over a much longer distance relative to other cells. Indeed, several toxicants that target intermediate filaments or MTs have their primary manifestation in neurons (Montine and Graham 2002). MTs are dynamic heteropolymers composed of α and β tubulin dimers that rapidly exchange with the pool of soluble tubulin as the key mechanism to their "growth" and transport of organelles within cells (Nogales 1999). Indeed, tubulin is the most abundant component of neuronal MTs, has at least 12 genetic variants, and is posttranslationally modified by several processes including, acetylation, tyrosinylation, glutamination, and phosphorylation (Westermann and Weber 2003). All of these modifications influence the ability of tubulin to polymerize or MTs to depolymerize, and in combination with MAPs such as tau determine the appropriate activity of tubulin and MTs.

Inheritance of mutations in the gene encoding multiple variants of tau, as well as one of three forms of neurofilament (NF), has been associated with several neurodegenerative diseases, including forms of Charcot-Marie-Tooth disease, Parkinson's disease, amyotrophic lateral sclerosis, and frontotemporal

dementia linked to chromosome-17 (FTDP-17) (Cairns, Lee et al. 2004). Elegant studies have shown that some forms of mutant tau are deficient in their ability to promote tubulin polymerization into MTs in vitro and also produce neurodegenerative features in vivo providing biochemical evidence that inheritance of at least some of these Tau mutations is sufficient to produce MT dysfunction and some aspects of neurodegeneration (Hong, Zhukareva et al. 1998; Barghorn, Zheng-Fischhofer et al. 2000; DeTure, Ko et al. 2002; Higuchi, Ishihara et al. 2002; del, Mederlyova et al. 2004; Krishnamurthy and Johnson 2004; Zhang, Higuchi et al. 2004). In contrast, we are unaware of any mutation in genes encoding tubulins that has been associated with neurodegeneration. These genetic abnormalities in a small number of patients establish the relevance of the cytoskeleton as a target for neurodegenerative diseases but do not enlighten the mechanisms by which abnormalities of cytoskeletal physiology may contribute to neurodegeneration in the many more patients who do not inherit mutations. In this regard, MTs and tau have received attention in sporadic or LOAD (Trojanowski and Lee 2002; Iqbal, Alonso Adel et al. 2005). Several morphologic and immunohistochemical studies have demonstrated changes consistent with MT dysfunction in LOAD, including synaptic vesicles that fail to reach the terminal compartment, vesicle accumulation in neuron soma, and increased mitochondrial elements in lysosomes (Scheff, DeKosky et al. 1990; Praprotnik, Smith et al. 1996; Terry 1996; Hirai, Aliev et al. 2001; Stokin, Lillo et al. 2005). Moreover, a few investigators have noted a reduction in stable MTs in brain specimens from patients with LOAD, even specimens obtained by biopsy

relatively early in the course of disease (Paula-Barbosa, Tavares et al. 1987; Hempen and Brion 1996; Cash, Aliev et al. 2003). In combination with these morphologic abnormalities, others have demonstrated clear biochemical abnormalities in tau from diseased regions of LOAD, viz., abnormal phosphorylation and increased ability to self-aggregate, as well as decreased activity in promoting tubulin polymerization in vitro.

While the etiology of MT dysfunction in FTDP-17 and other inherited tauopathies seem to derive, at least in part, from inherent dysfunction of mutant tau, it is less clear what the sequence of events is in the pathogenesis of LOAD. One possibility is that abnormal tau phosphorylation is unable to form MTs but instead aggregates as phosphorylated tau (P-tau). This shifts the dynamic state of MTs towards the soluble tubulin pool. An alternative is that other factors lead to MT dysfunction, resulting in liberation of tau and its subsequent abnormal phosphorylation and aggregation. While several studies have demonstrated the former by determining the activity of tau isolated from diseased regions of AD brain (Trojanowski and Lee 2002; Iqbal, Alonso Adel et al. 2005), we are unaware of any study that has directly investigated tubulin function independent of MAPs. What etiological factors may be causing MT disruption are not clear, but one suggestion is increased oxidative damage shown to occur in diseased regions of AD brain (Markesbery and Lovell 1998; Montine, Quinn et al. 2004; Smith, Nunomura et al. 2005). Increased products of oxidative stress are present in the cytosolic compartment of neurons in diseased regions of brain from patients with AD (Good, Werner et al. 1996; Smith, Sayre et al. 1996; Montine,

Kim et al. 1997; Montine, Olson et al. 1997; Sayre, Zelasko et al. 1997) some of which also have been shown to disrupt MTs (Neely, Sidell et al. 1999; Landino, Hasan et al. 2002; Roediger and Armati 2003; Allani, Sum et al. 2004) and even bind to tubulin (Gabriel, Miglietta et al. 1985; Miglietta, Olivero et al. 1991; Miglietta, Olivero et al. 1991; Neely, Sidell et al. 1999). We have shown previously that one of these, HNE, both binds to tubulin and disrupts neuronal MTs in cell culture (Neely, Sidell et al. 1999). HNE is a major aldehyde product of LPO that is elevated in diseased regions of brain and cerebrospinal fluid of patients with AD, and produces impaired performance on Morris water maze test following bilateral basal forebrain injection in rats (Lovell, Ehmann et al. 1997; Bruce-Keller, Li et al. 1998; Markesbery and Lovell 1998). However, other aldehydes, such as acrolein, also are produced in large quantities in diseased regions of brain from AD patients compared to controls (Calingasan, Uchida et al. 1999; Lovell, Xie et al. 2001). Here we tested the hypothesis that MTs are inherently dysfunctional in LOAD, and other neurodegenerative diseases, and determined whether this could be replicated by multiple products of oxidative damage.

3.2 Materials and Methods

Materials

HNE and HHE were synthesized as previously described (Neely, Sidell et al. 1999; Neely, Boutte et al. 2005). Acrolein was purified by distillation immediately before use. Unless otherwise indicated, materials used for cell

culture were from Invitrogen (Grand Island, NY) and all other chemicals were from Sigma (St. Louis, MO).

Human Brain Tissue

Human gray matter was obtained from the Neuropathology Core of the Alzheimer Disease Research Center at the University of Washington (UW) following appropriate informed consent, flash frozen in liquid nitrogen at time of autopsy, and stored at –80^{o}C. Use of human tissue was approved by the UW Institutional Review Board. Patients with AD were diagnosed during life probable AD and shown by neuropathologic examination that AD was the cause of their dementia (NIA 1997). Patients with inherited AD also were shown to harbor the N141I PS2 mutation. Patients with MCI were diagnosed with MCI during life according to accepted guidelines (Petersen, Doody et al. 2001) and were shown on neuropathologic examination to have low or intermediate levels of AD pathologic changes. Controls were evaluated within 2 years of death by neurological examination and psychometric testing with all results in the normal range and had age-related changes only by neuropathologic examination. Patients with FTDP-17 were diagnosed with frontotemporal dementia and shown to have the V337M or P301L mutation; neuropathologic examination showed changes typical of FTDP-17.

Tubulin polymerization assay

Taxol/GTP-stimulated tubulin polymerization assay using N2a cells was performed exactly as previously described (Neely, Boutte et al. 2005). This assay was adapted to human tissue by thawing each piece of frozen tissue at 1g/mL in ice-cold MES polymerization buffer supplemented with 1:5,000 protease inhibitor cocktail. Tissues were homogenized on ice at 20 second intervals

seconds using a pulsed probe sonicator at 1 pulse per second and then carried through the assay exactly as extracts from cells.

Immunoblot Analysis

Immunoblots were performed exactly as previously described (Neely, Boutte et al. 2005). Blots were probed with monoclonal α-tubulin antibody (1:1000, Sigma T5168), β-tubulin (1:1,000), Sigma T0198), or tau antibody (1:2,000, Dako Corporation A0024, Carpinteria, CA) and detected with 1:2,000 anti-mouse IgG-HRP antibody (Sigma, A3682). Blots were developed with chemilluminescence reagent (#NEL103001, NEN, Perkin Elmer Life Sciences, Boston, MA) and exposed to film (BioMax Light, Kodak or Blue-Lite film, ISC Bioexpress, Kaysville, UT).

Densitometry and Statistical analysis of microtubule polymerization

The percentage of extracted tubulin that was stimulated to polymerize was determined exactly as previously described (Neely, Boutte et al. 2005). Statistical analyses were performed with Graph Pad Prism (San Diego, CA).

3.3 Results

We determined the capacity of tubulin, extracted from human gray matter, to polymerize using the well-established assay of taxol/GTP-stimulated tubulin polymerization. It is important to note that taxol/GTP are a very potent stimuli of tubulin polymerization that act independently of MAPs (Schiff and Horwitz 1981; Neely, Boutte et al. 2005) . We utilized extracts of middle frontal gyrus (MFG) and superior and middle temporal gyri (SMTG), two regions involved by AD, in

patients with LOAD, mild cognitive impairment (MCI), and age-matched controls patients who died without disease in the CNS. Characteristics of these patients are presented in Table 1.

Table 1. Characteristics of Individuals Included in human brain tubulin polymerization. Data are mean ± SD for age and post mortem interval (PMI), ratio for sex, and mode for Braak stage and CERAD NP score (NIA 1997). ANOVA for age had P = 0.20 and for PMI had P = 0.23.

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Group	**n**	**age (year)**	**sex (F:M)**	**PMI (hour)**	**Braak Stage**	**NP Score**	**Mutation**
Control	10	75 ± 15	2:3	6 ± 3	I	0	None
MCI	4	88 ± 8	1:3	5 ± 2	II	Sparse	None
LOAD	12	76 ± 9	1:2	5 ± 2	VI	Frequent	None
AD/PS-2	4	71 ± 12	1:1	6 ± 4	VI	Frequent	N141I
FTDP-17	4	63 ± 3	1:1	8 ± 3	NA	NA	V337M, P301L

Representative western blots for α and β tubulins are presented in Figure 9. Average results for relative α and β tubulin in polymerized MT fractions are presented in Figures 10A and 10B, respectively. These results showed a significant difference among patient groups ($P < 0.0001$) but no difference between the two brain regions ($P > 0.05$), with reduced tubulin polymerization in AD patients compared to individuals with MCI or controls. Bonferroni-corrected posttests showed that the AD group was different from MCI and control ($P <$

0.001 for both comparisons), but that MCI and control were not different from each other ($P > 0.05$).

Figure 9.

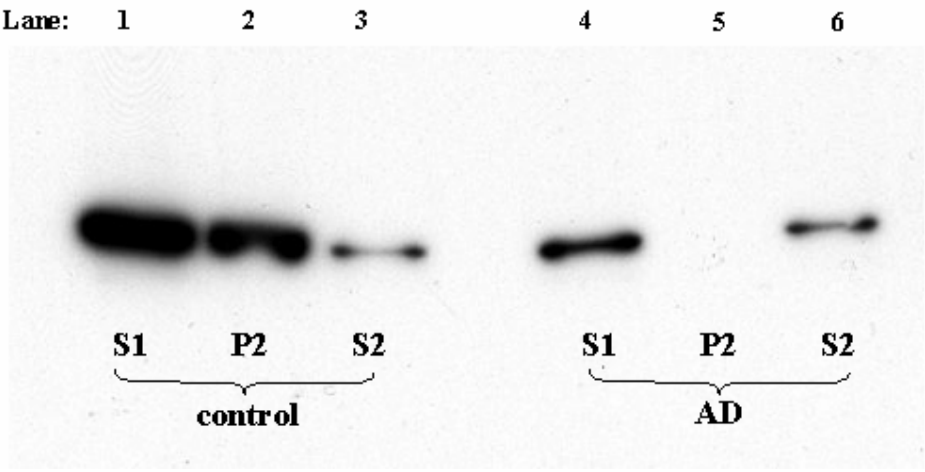


Figure 9. Tubulin polymerization in human tissue from LOAD is decreased compared to age matched controls. Lanes 1 and 4: S1, the first supernatant used for taxol/GTP polymerization, from age-matched controls and LOAD patients, respectively, before taxol/GTP stimulated polymerization. Lanes 2, 3, 5, and 6 are P2 and S2 from controls and LOAD patients. The western blot was probed with mouse monoclonal anti-α-tubulin.

Of the data presented in Figures 10A and 10B, we had paired measurements for MFG and SMTG from the same patient in a total of 38 samples (21 α-tubulin and 17 β-tubulin). Figure 10C shows the correlation for each pair of samples between the two brain regions. Overall, there was a highly significant linear relationship for taxol-stimulated tubulin polymerization between the two brain regions with the slope near unity ($P < 0.0001$; slope = 0.92 ± 0.09). Control and MCI values were broadly overlapping with 95% of samples having > 20% tubulin polymerization. Correlation for MCI (n=6) or Control (n=15) samples was similar to overall values ($P < 0.001$; slope = 1.04 ± 0.12 and 0.98 ± 0.17,

respectively), a result that indicates that the physiologic processes influencing tubulin polymerization in the absence of advanced AD are approximately the same in these two brain regions. Similar analysis of LOAD data revealed two categories: 9 of the 17 LOAD samples had no detectable polymerization, and thus are plotted at 0, 0 in Figure 10C; the other 7 samples showed no correlation between the two brain regions ($P > 0.5$, slope = -0.04 + 0.49). Although the number of samples is small, these data suggest possible regional differences in the pathologic process that underlie diminished tubulin polymerization from in a subset of LOAD brain extracts.

The variance in our taxol/GTP-stimulated tubulin polymerization data from LOAD patients suggests possible distinct subsets of patients within LOAD; therefore, we decided to investigate a known subset of AD patients distinct from LOAD, patients who had AD as a result of mutation in the PS-2 gene. For comparison, we included patients who had another type of inherited neurodegenerative disease that afflicts these regions of brain, frontotemporal dementia with Parkinsonism linked to chromosome-17 (FTDP-17). Characteristics of these patients are presented in Table 1. Results for taxol-stimulated α-tubulin are presented in Figure 11; similar results were achieved with β tubulin (not shown). Our results showed that both sets of diseased samples were significantly lower than controls and similar to LOAD. Again, tau distribution following taxol/GTP was approximately 50% with the pellet and supernatant fractions and was not significantly different between LOAD and FTDP-17 patients.

Figure 10.

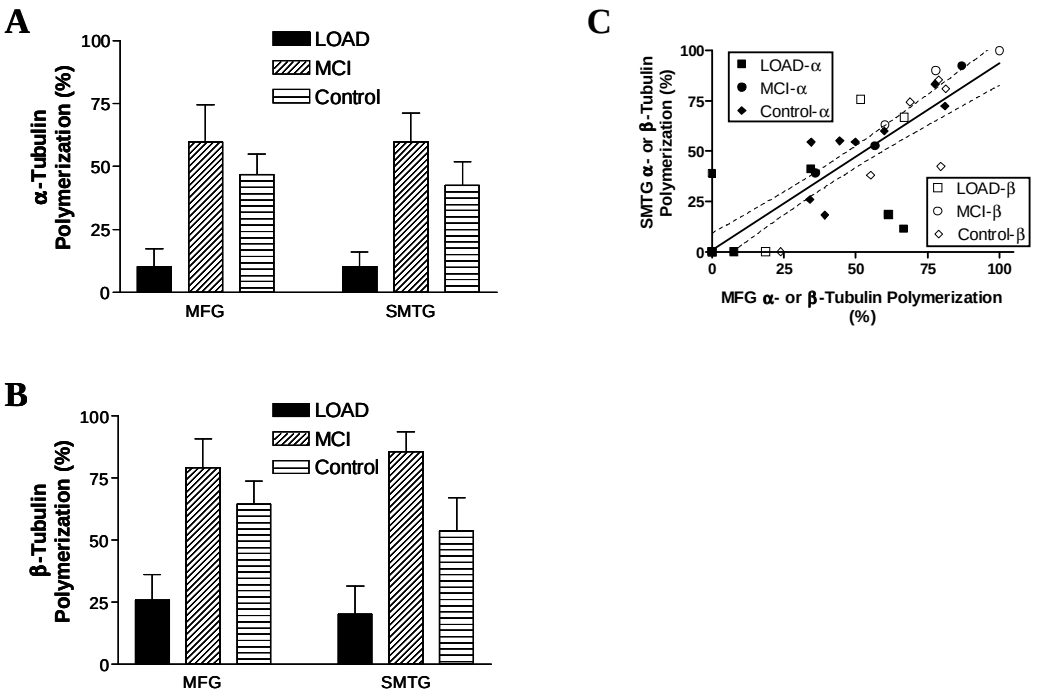


Figure 10. Taxol-stimulated α or β tubulin polymerization from middle frontal gyrus (MFG) and superior and middle temporal gyrus (SMTG) of patients who died with late onset Alzheimer's disease (LOAD), with mild cognitive impairment (MCI), or without disease in the central nervous system (Controls). Data are the percent of total tubulin that was stimulated to polymerize by taxol/GTP. Panels A and B have results for α and β tubulin polymerization stratified by group. Two-way ANOVA for α or β tubulin had $P < 0.0001$ for group, $P > 0.05$ for brain region, and $P > 0.05$ for interaction between these two terms. Panel C presents correlation of tubulin polymerization in the two regions of brain from the same individuals stratified by α or β tubulin and the three groups of individuals. Also shown are the best-fit line ($P < 0.0001$) and 95% confidence intervals for the all tubulin polymerization data from the two regions of brain.

Figure 11.

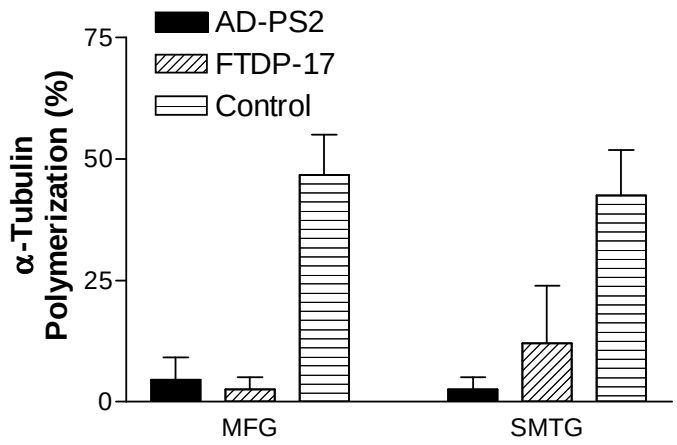


Figure 11. Taxol-stimulated α tubulin polymerization from middle frontal gyrus (MFG) and superior and middle temporal gyrus (SMTG) of patients who died with Alzheimer's disease caused by mutations in PS2, frontotemporal dementia with parkinsonism linked to chromosome-17 (FTDP-17), or without disease in the central nervous system (Controls). Data are the percent of total tubulin that was stimulated to polymerize by taxol/GTP. Two-way ANOVA had $P < 0.001$ for group, $P > 0.05$ for brain region, and $P > 0.05$ for interaction between these two terms.

We also probed these same samples for tau (Figure 12) and observed approximately 50% of tau in the pellet and the remainder in the supernatant; there was neither a difference in relative tau concentration in these two fractions among these groups of individuals (ANOVA had $P > 0.05$) nor was the pattern of tau immunoreactive bands different in this soluble extract of gray matter.

Figure 12.

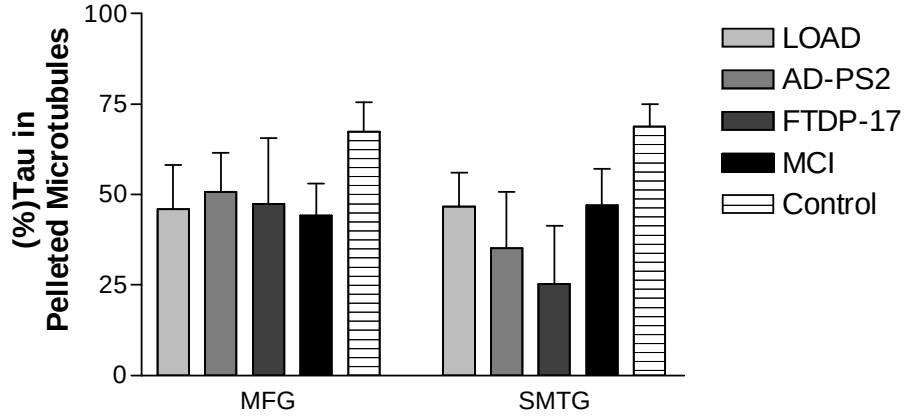


Figure12. Tau distribution of taxol-stimulated microtubule polymerization from middle frontal gyrus (MFG) and superior and middle temporal gyrus (SMTG) of patients who died with late onset Alzheimer's Disease (LOAD), Alzheimer's disease caused by mutations in PS2 (AD-PS2), frontotemporal dementia with parkinsonism linked to chromosome-17 (FTDP-17), mild cognitive impairment (MCI), or without disease in the central nervous system (Controls). Data are the percent of total tubulin that was stimulated to polymerize by taxol/GTP. Two-way ANOVA had $P < 0.001$ for group, $P > 0.05$ for brain region, and $P > 0.05$ for interaction between these two terms.

3.4 Discussion

Neuronal MTs are vulnerable targets that are morphologically abnormal in diseased regions of brain from patients with LOAD (Paula-Barbosa, Tavares et al. 1987; Hempen and Brion 1996; Cash, Aliev et al. 2003). Although there has been extensive investigation of tau, we are unaware of any study that has directly assessed tubulin function in LOAD. Here we tested the hypothesis that tubulin derived from LOAD gray matter was functionally impaired. We used taxol/GTP stimulation of tubulin polymerization because this drives tubulin polymerization independent of MAPs (Schiff and Horwitz 1981). We observed that on average, the capacity of taxol to stimulate tubulin polymerization was reduced in LOAD,

PS2-AD, and FTDP-17 but not individuals with MCI. These comparisons suggest that an inherent impairment of tubulin exists that prevents taxol binding or taxol-stimulated polymerization in these diseases. Moreover, in samples from patients classified as LOAD, we observed loss of correlation between brain regions as well as inter-individual heterogeneity in taxol-stimulated tubulin polymerization suggesting that pathogenic processes that are altering tubulin may not be uniform in this clinical/pathological entity. Given the known morphological abnormalities of MTs in LOAD, it is perhaps not surprising that we observed functional abnormalities in taxol-stimulated tubulin polymerization. Moreover, even though the etiologies differ, the pathogenic similarities between LOAD and PS2-AD again make for the reasonable hypothesis that abnormalities of tubulin function observed in one would be observed in the other. However, we were not expecting to observe abnormalities in taxol-stimulated tubulin polymerization in FTDP-17, a disease caused by abnormalities in tau that are not present in AD. From these findings, as well as preservation of tubulin function in samples from patients with MCI, we speculate the processes that are leading to tubulin dysfunction occur in intermediate or late stages of neurodegeneration and are common to LOAD, PS2-AD, and FTDP-17. There are a few (mechanistically overlapping) possibilities, including innate immune activation, excitotoxicity, and oxidative damage. We were especially interested in the latter because of the known ability of products of LPO to disrupt cellular MTs. Increased oxidative damage and subsequent LPO is now firmly established to be present in diseased regions of brain from patients with LOAD, PS2-AD, and FTDP-17. Several

groups have presented evidence, primarily histochemical and immunohistochemical, that neuronal cytoplasmic proteins are modified by aldehyde products of LPO in LOAD and other neurodegenerative diseases (Smith, Perry et al. 1996; Montine, Olson et al. 1997; Montine, Olson et al. 1997; Sayre, Zelasko et al. 1997; Montine, Reich et al. 1998; Velez-Pardo, Jimenez Del Rio et al. 1998; Calingasan, Uchida et al. 1999; Gerst, Siedlak et al. 1999). Moreover, at least two aldehyde products of lipid peroxidation, HNE and acrolein, are increased in tissue from LOAD patients compared to control (Lovell, Ehmann et al. 1997; Markesbery and Lovell 1998; Lovell, Xie et al. 2001). Similar to observations of N2a cells treated with pathologically relevant aldehyde concentrations, taxol stimulated tubulin polymerization was reduced in tissue from patients with LOAD, PS2-AD, and FTDP-17. Tubulin polymerization occurred at similar magnitudes across all three of these diseases.

There are important limitations to our work that need to be stressed. While impaired tubulin polymerization in tissue from patients with neurodegenerative disease was similar to what we observed in reactive aldehyde-exposed N2a cells, we have not demonstrated that reactive aldehydes from LPO are in fact accumulating on tubulin in these diseases. This is a very difficult issue. Since immunochemical methods lack the sensitivity and specificity to detect low levels of these adducts (Neely, Sidell et al. 1999), definitive demonstration of tubulin adducts will require cutting edge mass spectrometric techniques to discern among the various potential adducts that may form on different residues within α and β tubulin. Moreover, even if some of the different types of adducts from HNE,

HHE or acrolein are eventually demonstrated on tubulin from LOAD or these other diseases, this will not prove that these adducts alone are necessary or sufficient for the reduced capacity of tubulin to polymerize in these diseases. Indeed, this is an even more difficult problem that will require site-selective replacement of tubulin residues expressed in appropriate systems, such as cell culture or animal models.

In summary, we have demonstrated that exposure of neuronal cells to concentrations of aldehydes derived from lipid peroxidation at concentrations that occur under pathological states in vivo, results in irreversible reduction in the capacity of taxol to stimulate tubulin to polymerize and this effect was greatest for acrolein. We also observed similar irreversible loss of taxol-stimulated tubulin polymerization in samples from patients with LOAD, PS2-AD, and FTDP-17, but not individuals with MCI or controls. Our results show that modification of tubulin function, perhaps by aldehydes from LPO or other reactive molecules involved in oxidative damage, may contribute to intermediate or late stages in the pathogenesis of sporadic and inherited AD as well as FTDP-17.

CHAPTER IV

INCREASED FREQUENCIES OF β-III METHIONINE OXIDATION AND TAU LIPID PEROXIDATION ALZHEIMER'S DISEASE BRAIN DETERMINED BY MASS SPECTROMETRY AND P-MOD ANALYSIS

4.1 Introduction

AD is the most common form of dementia in the elderly and looms as a major public health problem in the coming decades. For these reasons, a large research effort is underway to identify key pathogenic steps in AD pathogenesis and develop directed therapies. A dominant biochemical feature of AD is the accumulation of detergent-insoluble protein, including sarkosyl-insoluble (SI) protein that can be extracted by highly chaiotropic means, such as partial solubility in formic acid (FS) (Kakizuka 1998; Trojanowski and Lee 2000; Tabner, Turnbull et al. 2001; Ingelsson and Hyman 2002; Hashimoto, Rockenstein et al. 2003). Two proteins which undergo this transition from normal solubility to SI/FS are Aβ and tau, the major protein constituents of senile plaques and NFTs, respectfully. However, several other proteins also are present in the SI/FS fraction in AD, such as tubulins and glial fibrillary acidic protein (GFAP), the intermediate filament of astrocytes (Woltjer, Cimino et al. 2005). SI/FS tubulins may be especially important because highly dynamic exchange between soluble and polymerized tubulin heterodimers is critical to MT function, and MT dysfunction has been proposed as one of the earliest events in AD pathogenesis (Stokin, Lillo et al. 2005).

Despite the potential importance of this transition of proteins from normal solubility to SI/FS, relatively little is known about the mechanisms that underlie this change. Recent elegant studies have demonstrated biophysical mechanisms by which Aβ forms insoluble fibrils *in vitro* (Walsh, Hartley et al. 1999; Arimon, Diez-Perez et al. 2005; Kirkitadze and Kowalska 2005; Petkova, Leapman et al. 2005), and it seems likely that inherent properties of some protein structures and protein-protein interactions will be key to determining which proteins transition to abnormal and perhaps pathologic insolubility. Another proposed mechanism for transition to SI/FS is post-translational modification of protein by oxidative damage (Butterfield, Castegna et al. 2002; Reynolds, Berry et al. 2005). Indeed, oxidative damage to protein, lipid, and nucleic acid has been repeatedly associated with diseased regions of brain from patients who died with AD and cerebrospinal fluid from patients with early AD or even MCI (Smith, Carney et al. 1991; Lyras, Cairns et al. 1997; Markesbery 1997; Smith, Richey Harris et al. 1997; Keller, Schmitt et al. 2005), a prodromal condition that commonly progresses to AD (Morris and Price 2001; Morris, Storandt et al. 2001). Numerous studies of human tissue have employed antibodies to localize oxidatively modified proteins; broadly, these studies have localized oxidation, glycation, nitration, and LPO protein adducts to neuron cytosolic proteins and sometimes to NFTs in AD brain (Smith, Sayre et al. 1998; Aksenova, Aksenov et al. 1999). In addition to these patient-oriented studies, transgenic mouse models of AD also have been reported to show increased indices of oxidative damage, even before the deposition of Aβ-immunoreactive plaques (Pamplona, Dalfo et

al. 2005). While the sources of oxidative damage in AD are not entirely resolved, they include the potentially partially overlapping processes of Aβ peptide formation, innate immune activation, excitotoxicity, and mitochondrial dysfunction.

An elegant series of experiments have coupled 2-dimensional gel electrophoresis and immunochemical detection of proteins with carbonyl modifications studied by tandem mass spectrometry (MS-MS) to obtain a broad view of proteins labile to this form of oxidative modification in AD patients (Castegna, Aksenov et al. 2002; Castegna, Aksenov et al. 2002; Castegna, Aksenov et al. 2002; Korolainen, Goldsteins et al. 2002) and in rats following intracerebral injection of $A\beta_{42}$ (Boyd-Kimball, Sultana et al. 2005). Another recent study determined the amount of five different amino acid modifications in frontal cortex from AD patients by quantifying modified amino acids by gas chromatography (GC)-MS (Pamplona, Dalfo et al. 2005). While both of these approaches have provided insight into the pathogenesis of protein modification in AD, both studies examined proteins extracted into aqueous buffer, *i.e*., proteins with normal solubility. Indeed, no study we are aware of has associated oxidative protein modifications in AD with transition to detergent-insolubility, mapped protein modifications, or concentrated on neuronal cytoskeletal proteins, proposed to be the site of earliest dysfunction in AD pathogenesis.

A scoring algorithm for spectral analysis (SALSA), which performs automated pattern recognition for peptide modifications in MS-MS data, has been used to identify oxidative protein modifications *in vitro* (Badghisi and Liebler

2002; Liebler, Hansen et al. 2002), including $A\beta_{40}/Cu^{2+}$-induced histidine and methionine oxidation (Schiewe, Margol et al. 2004). A related algorithm, called P-MOD identifies and maps modifications to peptide sequences using MS-MS data (Hansen, Davey et al. 2005) and provides probability-based estimates of the quality of the matches. Here we combine well-established extraction methods with liquid chromatography (LC)-MS-MS and P-MOD analysis to identify and map oxidative modifications to four cytoskeletal proteins that are associated with the biochemical abnormality of detergent insolubility or accumulation into NFTs.

4.2 Methods

Human Brain Tissue

Use of human tissue was approved by the University of Washington (UW) Institutional Review Board. Human brain samples were obtained from the Neuropathology Core of the Alzheimer Disease Research Center (ADRC) at UW following appropriate informed consent, flash frozen in liquid nitrogen at time of autopsy, and stored at –80°C. Patients with AD were volunteers in the UW ADRC where they were diagnosed during life with probable AD and shown by neuropathologic examination to have AD (The National Institute on Aging and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's 1997). Control individuals also were volunteers in the UW ADRC, were never diagnosed during life with disease of the CNS, and had age-related changes only by neuropathologic examination.

Extraction and Dissection

Tissue was first extracted with Buffer A (10 mL/g tissue in 10mM Tris, 1mM EGTA, 1mM DTT, 10% sucrose, pH 7.5) as previously described (Wang, Woltjer et al. 2005; Woltjer, Cimino et al. 2005). Following centrifugation at 26, 500g for 15 minutes at 4°C, the insoluble pellet was sequentially extracted with Buffer A plus 1% triton, Buffer A plus 1% n-laurylsarcosyl, and 70% formic acid (FA) exactly as we have done previously (Woltjer, Cimino et al. 2005). Laser capture microdissection (LCM) of NFTs was performed exactly as previously described by us (Wang, Woltjer et al. 2005). Briefly, hippocampus obtained at autopsy was embedded in cryoprotective compound, frozen, and stored at –80°C. Frozen sections were cut at 10 micron thickness, fixed, permeabilized, probed with mouse monoclonal anti-tau antibody (Tau-2, Sigma, St. Louis, MO; 1:250), and LCM of tau-2-immunoreactive structures with the size and shape of NFTs was performed in CA1 sector. A total of approximately 2000 NFTs were pooled and extracted with 70% FA. These protein preparations were repeated three times for both extractions and twice for LCM of NFTs. Proteins extracted in Buffer A, the SI/FS fraction, and LCM-obtained proteins were desalted, alkylated with iodoacetamide, cleaved with trypsin, and prepared for LC-MS-MS exactly as previously described (Wang, Woltjer et al. 2005; Woltjer, Cimino et al. 2005).

Mass Spectrometry

Each of the three samples of digested peptides from Buffer A and SI/FS fractions and both of the samples from LCM NFTs were separately purified with a

C18 solid phase extraction column (Oasis^{R} MCX, Milford, MT), separated by a two dimensional microcapillary high performance LC system, a strong cation-exchange column with two alternating reversed-phase C18 columns (10 cm X 180 µm), followed by analysis with MS-MS (Thermo Electron, San Jose, CA) exactly as previously described (Wang, Woltjer et al. 2005; Woltjer, Cimino et al. 2005). MS-MS data were searched against the International Protein Database using SEQUEST. The sensitivity and specificity of protein identification were determined by Peptide Prophet and Protein Prophet software (Wang, Woltjer et al. 2005; Woltjer, Cimino et al. 2005; Zhang, Goodlett et al. 2005).

P-MOD analysis

Tryptic peptide fragments 5-30 amino acids in length, with and without internal lysines, were generated *in silico* using the following protein sequences obtained from NCBI/ENTREZ or SWISS PROT databases: α-III tubulin (Swiss Prot Q71U36), β-III tubulin (Swiss Prot Q13509), PHF tau (Swiss Prot P10636), and GFAP (Swiss Prot P14136). Each file of MS-MS data (three replicates for Buffer A, three replicates for SI/FS, and two replicates for LCM NFTs) was evaluated separately by P-MOD searching for trypsin-cleaved proteins with and without missed lysines, and the results from these replicate evaluations were then combined. Using the Associate of Biomolecular Research Facilities (ABRF) Delta Mass (http://www.abrf.org/index.cfm/dm.home) and Unimod (http://www.unimod.org/) databases, we tentatively assigned structures to the mass shifts identified and mapped by P-MOD. The average (± standard

deviation) error rate in mass shift determination was 2.5 $\pm$ 0.2% of all peptides included in this study. Modification frequency was estimated as the number of modified amino acids that met the above criteria divided by the total number of times those amino acids were present in that specific peptide. χ-squared tests (GraphPad Prism, San Diego, CA) were performed to assess the statistical significance of modification frequency for each protein in Buffer A, SI/FS, and LCM NFT preparations; since we performed repeated χ-square analyses, we accepted as significant only those with $P < 0.01$.

Cyanogen Bromide Cleavage and Western Blotting

The SI/FS fraction from temporal cortex from AD patients and the 70% FA extract from control temporal cortex were dried, resuspended in water, and protein concentration determined using the Biorad Dc Reagent Kit (Biorad, Hercules, CA). 50µL of each was dried under vacuum and then resuspended in either 500 µL 70% FA or 100 mM CNBr plus 70% FA per 12.4 µg total protein; this achieved a 100-fold molar excess of CNBr to tubulin that was estimated to be ~10% total cellular protein (McLaughlin, Zemlan et al. 1997; Kaiser and Metzka 1999; Hollemeyer, Heinzle et al. 2002). Samples were digested overnight, dried, and resuspended in Laemmli sample buffer containing 200 mM DTT for separation by SDS PAGE using Tris-Tricine Ready Gels (Biorad, Hercules, CA). Western blots were performed exactly as previously described (Neely, Boutte et al. 2005) using monoclonal anti-α-tubulin antibody (Sigma Chemical Co., St. Louis, MO) at 1:1000 dilution or monoclonal anti-β-III tubulin

antibody (Covance Research Products, Babco, CA) at 1:1000 dilution. Outcomes from Western blots were digitized and band density integrated with ImageJ software (National Institutes of Health, Bethesda, MD); statistical comparison was made by two-way analysis of variance (ANOVA) (Graph-Pad Prism).

4.3 Results

Extracts were prepared from temporal cortex, a region of brain affected by processes of AD, and pooled from 5 patients before LC-MS-MS to limit idiosyncratic differences among individuals (Zhang, Goodlett et al. 2005). Information on AD patients from whom tissue was obtained is presented in Table 2. For each patient, we prepared two serial fractions from the same piece of tissue: a fraction soluble in detergent-free Buffer A and a SI/FS fraction (Wang, Woltjer et al. 2005; Woltjer, Cimino et al. 2005). There are two major advantages to comparing normally soluble with insoluble protein from the same AD tissue. First, direct comparison of SI/FS protein between AD patients and controls is confounded because carefully established control individuals have 6-fold less SI/FS protein relative to AD patients, and what is present in SI/FS extracts from controls has different protein constituents than extracts from AD patients (Woltjer, Cimino et al. 2005). Second, important issues related to protein changes from co-morbid conditions, agonal state, delay in procurement, and slight variation in dissection, freezing, and extraction of tissue are removed because the same piece of tissue was used to prepare both fractions. For comparison, a third

fraction was prepared by FA extraction of NFTs obtained by LCM of AD hippocampal sector CA1 (Wang, Woltjer et al. 2005).

Table 2. Information on individuals whose tissue was used in P-MOD experiments. AD tissue for CNBr cleavage was the same as that used in extraction studies for P-MOD.

	Extraction for P-MOD	**LCM of NFTs for P-MOD**	**CNBr Cleavage**
Diagnosis	**AD**	**AD**	**Control**
n	5	4	3
Age (years)	80 $\pm$ 1	81 $\pm$ 4	84 $\pm$ 4
F:M	3:2	1:1	2:1
PMI (hr)	3.4 $\pm$ 0.8	3.7 $\pm$ 1.2	4.2 $\pm$ 1.1
CERAD NP	Moderate or Frequent	Moderate or Frequent	None or Sparse
Braak	VI	VI	0 to II

Extracts were digested by trypsin and prepared for (LC)-MS-MS as previously described (Wang, Woltjer et al. 2005; Woltjer, Cimino et al. 2005); even the extensively posttranslationally modified protein in the LCM NFT fraction is virtually completely digested by trypsin as assessed by SDS-PAGE and silver stain (Wang, Woltjer et al. 2005). We focused on 4 different cytoskeletal proteins identified in each preparation by > 2 unique peptides and a Protein Prophet probability score of 1.00: neuron-enriched α-III tubulin (Hall and Cowan 1985; Miller, Naus et al. 1987) and β-III tubulin (Lee, Rebhun et al. 1990; Lee, Tuttle et

al. 1990; Lu, Jones et al. 2003) that are also constituents of NFTs (Wang, Woltjer et al. 2005), neuron-enriched tau that is the major constituent of NFTs (Harrington, Mukaetova-Ladinska et al. 1991), and astrocyte-enriched GFAP (Eng, Ghirnikar et al. 2000; Messing and Brenner 2003). We used P-MOD to analyze the MS-MS data from these four proteins in three different preparations for 10 mass shifts on appropriate amino acids that are associated with oxidative and nitrative damage (Table 3). As a confirmation of our approach, we also examined phosphorylation (+80 amu) of tau because of the expected increase in tau phosphorylation in the SI/FA and NFT preparations compared to Buffer A-soluble tau (Alonso, Grundke-Iqbal et al. 1996; Iqbal, Alonso Adel et al. 2005). A total of 29,846 peptides were evaluated by P-MOD in this study; the total number of peptides analyzed per protein, the median number of peptides analyzed per tryptic fragment, and the range of peptides analyzed per tryptic fragment are presented in Table 4.

Modification frequency (the number of modified labile amino acids divided by the total number of labile amino acids) was estimated as described in Methods. We used χ-squared analysis of modification frequency in the three sample preparations (with $\rho = 0.01$ because of repeated tests) to assess whether modification frequencies were changing significantly from Buffer A to SI/FS to LCM NFT fractions. As expected, the frequency of tau phosphorylation increased significantly in SI/FA and NFT fractions compared to Buffer A-extracted tau from AD temporal cortex. Of the 48 possible combinations of 10 oxidative or nitrative modifications on 4 different proteins, four others also had χ-

squared tests with $P < 0.01$; these were: β-III tubulin methionine oxidation, α-III tubulin nitration, and tau adduction by HHE and HNE. Table 5 presents a summary statistic, the sum of modification frequencies for all labile amino acids in the entire protein. Of the modifications with significantly increased frequency, β-III tubulin methionine oxidation, sulfone and sulfoxide combined, was the most prevalent with over 80% of all methionine residues within this protein oxidized to their corresponding sulfoxide or sulfone. Figure 13 maps the distribution of methionine oxidation frequency in β-III tubulin, and Figure 14 maps the distribution of HNE and HHE adducts on tau, in each of the three preparations.

We pursued verification of the most prevalent adduct, methionine oxidation on β-III tubulin, through independent means by determining efficiency of CNBr-mediated cleavage, which is blocked by oxidized methionines (Shechter, Burstein et al. 1975; Villa, De Fazio et al. 1989; Hollemeyer, Heinzle et al. 2002). For comparison, we used SI/FS extract of temporal cortex from the same 5 AD patients whose tissue was used in P-MOD analysis and compared that to extracts of temporal cortex from three individuals who died without clinical evidence of neurological disease and who had age-related changes only in brain by neuropathologic examination; characteristics of these individuals are presented in Table 2. Since the total SI extractable protein in controls is very small (Woltjer, Cimino et al. 2005), control tissue was extracted directly with 70% FA. Proteins extracted from controls and AD patients were subjected to cleavage with CNBr, separated by SDS-PAGE and then probed by Western blots with antibodies against α and β-III tubulin; antibodies specific to neuron-enriched

α-III tubulin are not available (Figure 15). Our results showed that CNBr treatment cleaved virtually all α and β-III tubulin extracted from control tissue, while cleavage of β-III tubulin was selectively and significantly reduced in extracts from AD tissue ($P \leq 0.01$).

Table 3. Mass shifts, relevant amino acids, and the corresponding proposed oxidative and nitrative modifications investigated with P-MOD.

Mass Shift	**Relevant Amino Acid**	**Proposed Adduct/Modification**
-64	M	Methane sulfenic acid
-27	R	Arginine oxidation to glutamic semialdehyde
+16 or +32	M	Methionine Oxidation
+46 or +62	F, T, Y, W	Nitration
+48	C	Cysteic acid
+56	K	Carboxymethyl-lysine, Glyoxal, or Acrolein
+72	C, K, Q, R	Methylglyoxal, Carboxyethyl-lysine (K only)
+113	C, H, K	4-hydroxyhexenal (HHE)
+156	C, H, K	4-hydroxynonenal (HNE)

Table 4. Peptide hits evaluated by P-MOD for each protein in each preparation. * One peptide was not observed for these proteins in the NFT faction.
^ Five peptides were not observed for tau in the NFT fraction.

		α-III Tubulin	**β-III Tubulin**	**Tau**	**GFAP**
Buffer A	*Total # hits*	3354	3118	2675	3937
	Median # hits/peptide	115	131	52	88
	Range of hits /peptide	24 - 439	27 - 332	4 - 288	19 - 289
SI/FS	*Total # hits*	3665	3086	1919	4166
	Median # hits/peptide	140	135	43	87
	Range of hits /peptide	36 - 329	10 - 322	2 - 265	17 - 261
NFT	*Total # hits*	936	361	1020	1609
	Median # hits/peptide	29	14	17	32
	Range of hits /peptide	1 - 99	0* - 52	0* - 190	0* - 114

Table 5. Sum of modification frequencies for those proteins with *P < 0.01 for χ-squared test of modification frequency among the three tissue preparations. For example, 119 amino acids contained within tau are labile to phosphorylation (S, T, and Y). If every time a specific amino acids was observed it had a mass shift of +80 ascribed by P-MOD, then the frequency of modification at that amino acid would be 1.00. If every S, T, and Y within tau had an individual modification frequency of 1.00, then the sum for the entire protein would be 119.00. Number (#) indicates total possible amino acids labile to the proposed modification.

Proposed Modification	**Preparation**	**α-III tubulin**	**β-III -tubulin**	**tau**	**GFAP**
Phosphorylation	Buffer A SI/FS NFT (#)	-- -- -- --	-- -- -- --	3.57 23.80 21.42* (119)	-- -- -- --
Methionine Oxidation	Buffer A SI/FS NFT (#)	0.02 0.00 0.03 (6)	0.52 5.16 11.7* (14)	0.16 1.00 1.94 (8)	0.15 0.20 0.00 (11)
Nitration	Buffer A SI/FS NFT (#)	12.65 11.00 2.20* (55)	4.90 0.00 0.98 (49)	6.38 3.48 0.58 (58)	6.08 14.44 10.26 (38)
HHE	Buffer A SI/FS NFT (#)	0.41 0.50 0.08 (30)	0.05 0.07 0.04 (24)	0.68 11.00 0.16* (84)	1.73 0.36 0.06 (31)
HNE	Buffer A SI/FS NFT (#)	3.90 0.00 3.00 (30)	0.00 0.00 0.00 (24)	1.68 11.76 2.52* (84)	0.93 0.00 4.03 (31)

Figure 13.

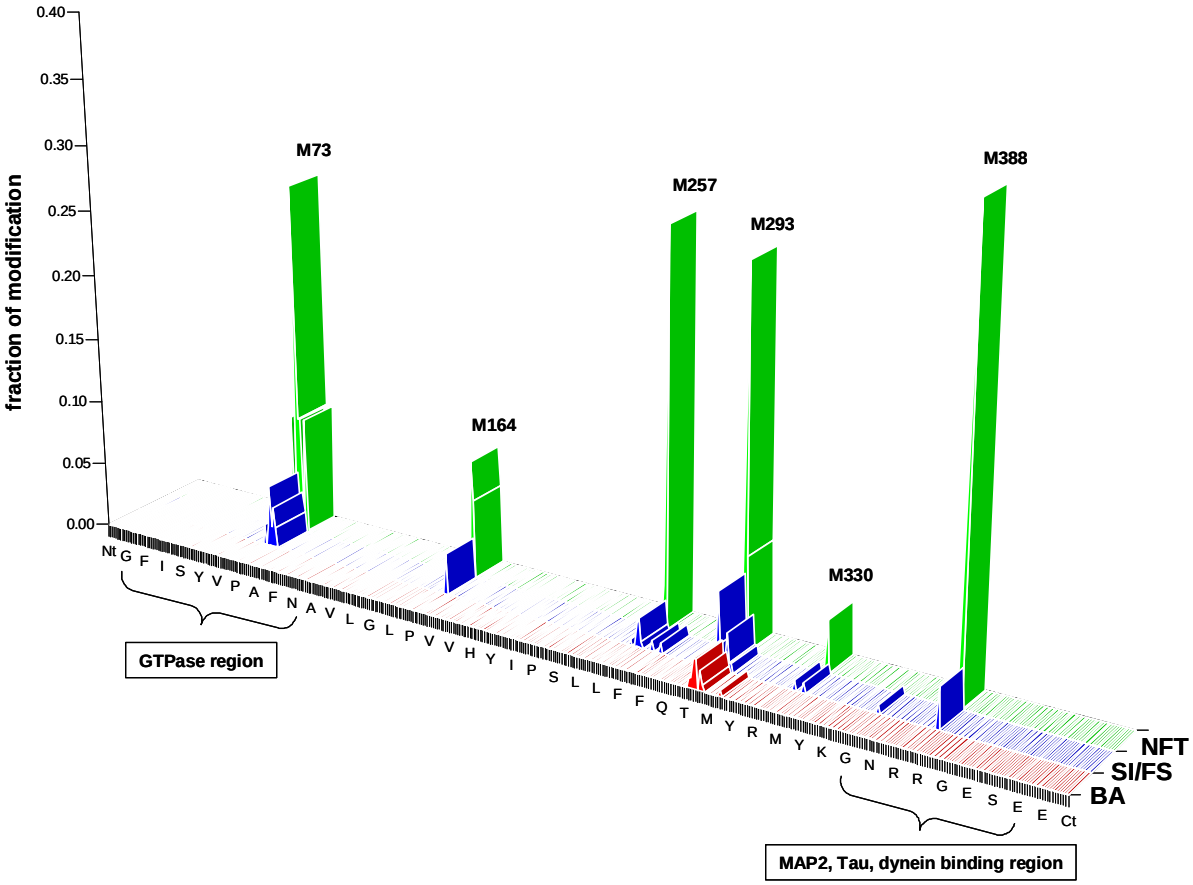
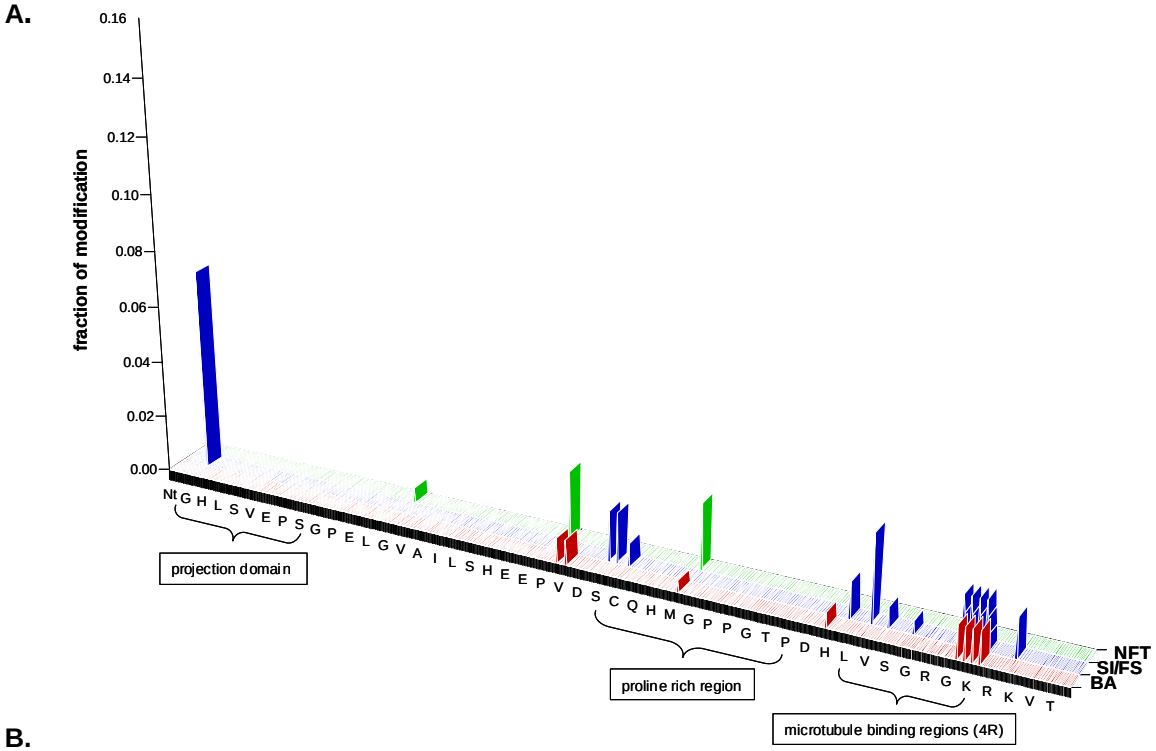


Figure 13: Map of +16 or +32 amu shifts on methionines for tryptic peptides from β-III tubulin in the three tissue extracts. Human temporal cortex from patients with AD was serially extracted into Buffer A (BA) or sarkosyl insoluble/70% formic acid soluble (SI/FS) fractions. A third sample, laser captured NFTs, was extracted directly into 70% formic acid. All samples were analyzed by liquid chromatography with tandem mass spectrometry and then evaluated by P-MOD. Data are presented as amino acid sequence from N-terminal to C-terminal with every 10th residue shown on the x-axis, tissue preparation on the y-axis, and frequency of methionine sulfoxide (M+16) and methionine sulfone (M+32) on the z-axis. Also designated are some functional domains. χ-squared test for methionine oxidation *vs.* tissue preparation had $P < 0.01$.

Figure 14.



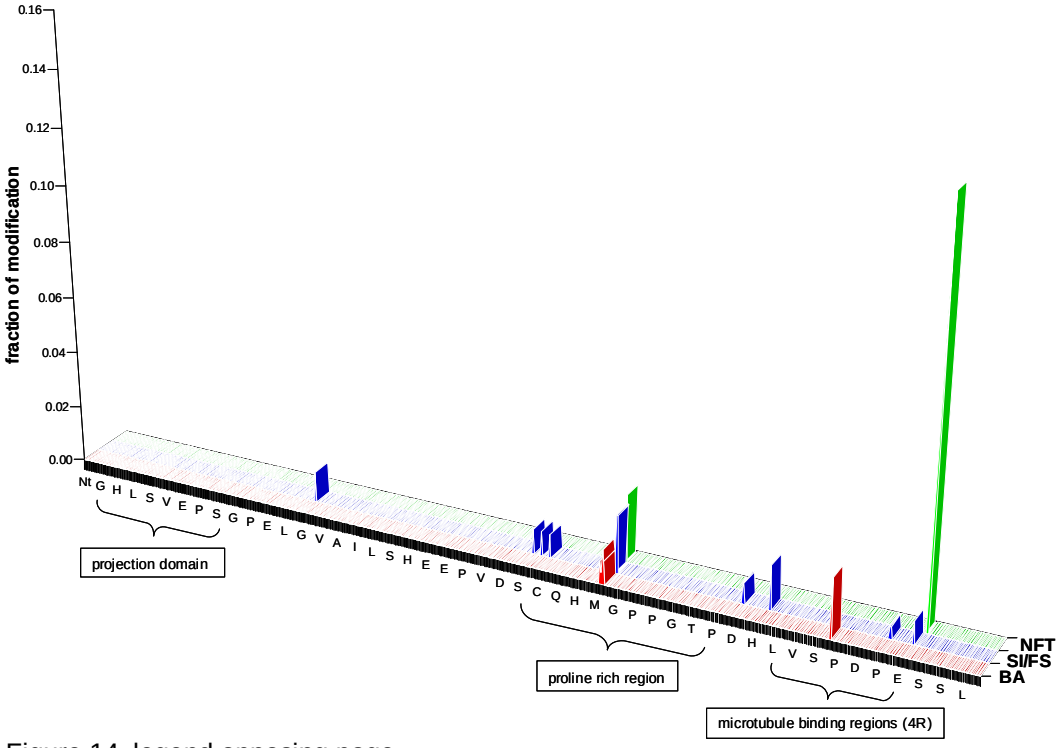


Figure 14. legend opposing page

Figure 14: Map of +113 amu (A) (proposed 4-hydroxyhexenal (HHE) Michael adduct) and +156 amu (B) (proposed 4-hydroxynonenal (HNE) Michael adduct) shifts on C, H, and K for tryptic peptides from tau in the three tissue extracts. Human temporal cortex from patients with AD was sequentially extracted into Buffer A (BA) or sarkosyl insoluble/70% formic acid soluble (SI/FS) fractions. A third sample, laser captured NFTs, was extracted directly into 70% formic acid. All samples were analyzed by liquid chromatography with tandem mass spectrometry and then evaluated by P-MOD. Data are presented as amino acid sequence from N-terminal to C-terminal with every 16^{th} residue shown on the x-axis, tissue preparation on the y-axis, and frequency of proposed HHE or HNE adducts on the z-axis. Functional domains are also designated. χ-squared test for HHE or HNE *vs.* tissue preparation both had $P < 0.01$.

Figure 15.

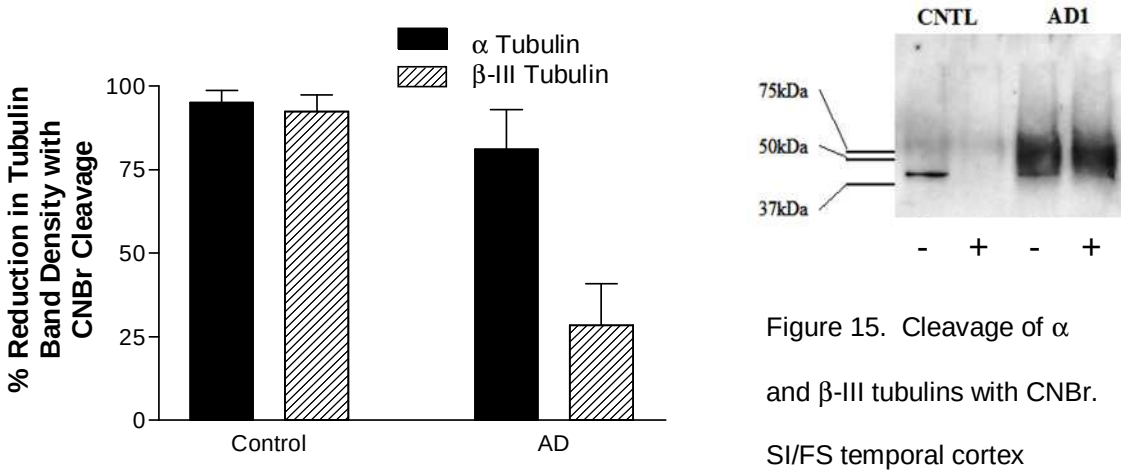


Figure 15. Cleavage of α and β-III tubulins with CNBr. SI/FS temporal cortex fraction from the same AD patients analyzed by P-MOD and FS temporal cortex fraction from three control individuals were subjected to cleavage by CNBr, separated by SDS-PAGE, and probed with α or β-III tubulin antibody. Data are presented as the percent change from samples run in parallel that were not incubated with CNBr. Two-way ANOVA had $P < 0.05$ for α *vs.* β-III tubulin, $P < 0.01$ for control *vs.* AD, and $P < 0.05$ for interaction between these two terms. Bonferroni-corrected post-tests had $P < 0.01$ for AD but $P > 0.05$ for controls. Inset: Representative Western blot of control (CNTL) and AD extracts incubated with (+) or without (-) excess CNBr and probed with β-III tubulin antibody.

Figure 16.

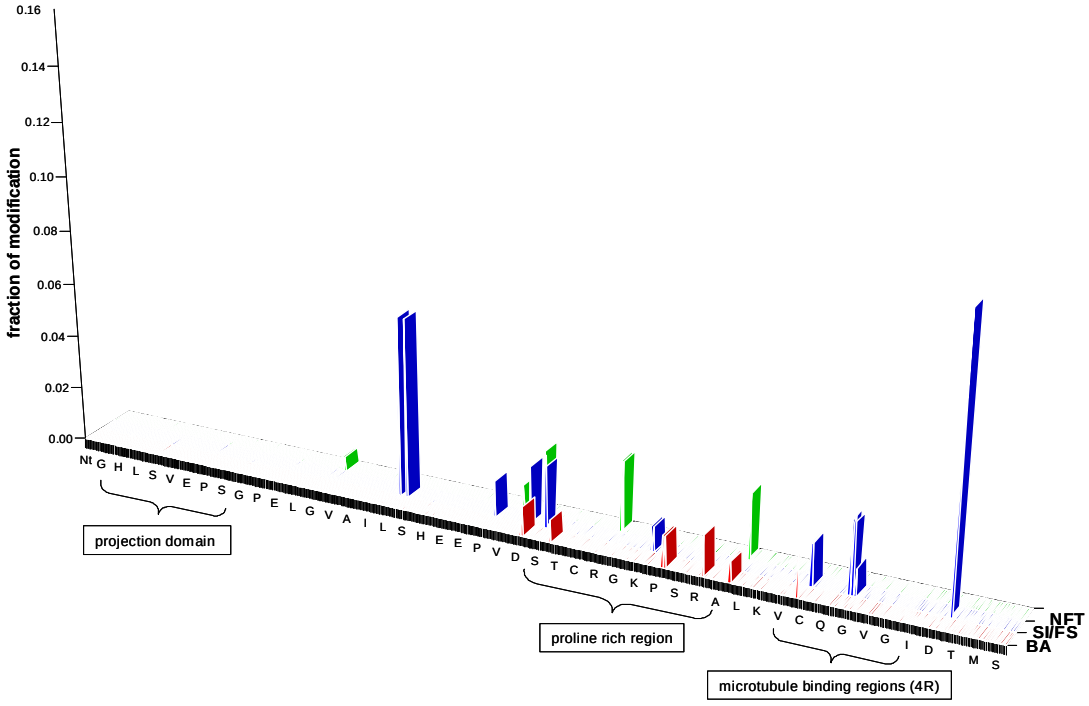


Figure 16**.** Map of +80 amu (proposed phosphorylation) shifts on S, T, and Y for tryptic peptides from tau in the three tissue extracts. Human temporal cortex from patients with AD was sequentially extracted into Buffer A (BA) or sarkosyl insoluble/70% formic acid soluble (SI/FS) fractions. A third sample, laser captured NFTs, was extracted directly into 70% formic acid. All samples were analyzed by liquid chromatography with tandem mass spectrometry and then evaluated by P-MOD. Data are presented as amino acid sequence from N-terminal to C-terminal on the x-axis, tissue preparation on the y-axis, and frequency of proposed phosphate adducts on the z-axis. Functional domains are also designated. X-squared test for HHE or HNE *vs.* tissue preparation both had $P < 0.01$.

4.4 Discussion

Here we used P-MOD, a recently developed software and algorithm that identifies and maps peptide modifications by analyzing MS-MS data, to evaluate three different protein fractions obtained from human temporal cortex affected by AD: a biochemically normal protein fraction that was extracted into Buffer A, a pathological protein fraction that was insoluble in ionic detergents (SI/FS), and proteins associated with NFTs, a histopathologic hallmark of AD. We focused our analysis on neuron-enriched tubulins and tau, as well as GFAP as an internal control, because neuronal cytoskeletal dysfunction has been proposed as one of the earliest abnormalities in AD pathogenesis (Stokin, Lillo et al. 2005). We further focused our analysis on 10 mass shifts characteristic of oxidative and nitrative modifications because results from several model systems have shown that these modifications of tubulins and tau may be pathophysiologically relevant and lead to abnormal protein-protein interactions as well as neuronal cytoskeletal collapse (Horiguchi, Uryu et al. 2003; Reynolds, Berry et al. 2005; Zhang, Xu et al. 2005). We also investigated tau phosphorylation because of the strongly anticipated outcome, based upon many biochemical and immunochemical studies (Kopke, Tung et al. 1993; Alonso, Zaidi et al. 1994; Alonso, Grundke-Iqbal et al. 1996; Buee, Bussiere et al. 2000), that its frequency would be increased in the pathological preparations compared to Buffer A extracts.

P-MOD evaluated over 28,000 peptides in this study. Our results demonstrated that among the neuron-enriched cytoskeletal proteins investigated, detergent insolubility of β-III tubulin was associated with selective methionine

oxidation, to the exclusion of its heterodimeric partner, and detergent-insoluble tau contained significantly increased modifications by LPO products. It is important to stress, perhaps most so for methionine oxidation, that similar changes were not observed on GFAP, an astrocyte-enriched protein that was extracted simultaneously. We confirmed by independent means that CNBr cleavage of β-III tubulin was selectively decreased in AD tissue, a result consistent with our P-MOD analysis. Tau phosphorylation was an expected result, although never previously demonstrated by this method, and a further validation of our approach. Many reports have demonstrated bulk increased levels of methionine oxidation and LPO in AD tissue by biochemical methods and localization of these changes primarily to neurons by immunohistochemistry (Montine, Kim et al. 1997; Sayre, Zelasko et al. 1997; Montine, Reich et al. 1998; McKracken, Graham et al. 2001; Dalle-Donne, Rossi et al. 2002; Stadtman, Van Remmen et al. 2005); these results are consonant with our protein mapping study.

While we think it is informative to include NFT-associated proteins in our analysis, there are limitations to interpreting these data. First, unlike Buffer A and SI/FA fractions, NFTs were captured from different pieces of tissue leaving the sample vulnerable to the potential sources of variance noted earlier. Moreover, proteins obtained by LCM will include not only structurally abnormal proteins present in NFTs, but also unknown amounts of protein with normal solubility since this dissected tissue was extracted directly into FA because of it small mass. Finally, the biochemical abnormalities of some NFT-associated

proteins are likely to be among the most extreme and may confound analysis by P-MOD. These considerations coupled with the relatively smaller amount of protein analyzed from the NFT LCM fraction (with consequent incomplete peptide coverage, see Table 3) likely make these the least accurate data obtained in these experiments. We observed a lower frequency of tau phosphorylation, β-III tubulin methionine oxidation, and tau modification by HHE and HNE in LCM NFT compared to SI/FS extracts. Our data showed that these modifications were detectable in these proteins in the LCM NFT preparation and mapped in a manner similar to the SI/FA fraction; however, we are cautious about comparing the relative frequency of protein modifications between these two preparations given the limitations noted above.

Interestingly, our data showed that nitrative modification of these cytoskeletal proteins changed significantly across sample preparations only for β-III tubulin and actually decreased from Buffer A to the SI/FS fraction. Still further reduction in nitrative frequency was observed in LCM NFT fraction. Nitrative modification of protein does occur as part of normal physiology, including α-tubulin (Bolan, Gracy et al. 2000; Greenacre and Ischiropoulos 2001; Bisig, Purro et al. 2002); however, bulk biochemical and immunohistochemical studies have concluded that AD is associated with an increase in nitrative modification of protein (Smith, Richey Harris et al. 1997; Eiserich, Estevez et al. 1999; Castegna, Thongboonkerd et al. 2003). We are aware of no study that has specifically investigated α-III tubulin in this regard and so are left with the

possibility that part of AD pathogenesis may be increased nitrative modification of some proteins and decreased nitrative modification of others.

Not only did our analyses detect selective accumulation of oxidative modifications on some of these biochemically abnormal neuron-enriched cytoskeletal proteins, but we also demonstrated that these modifications were non-randomly distributed within the selectively modified protein. The mechanisms underlying this selective distribution of oxidative protein modification in AD are not clear but likely represent a complex interaction among generation of the oxidizing agent, the inherent susceptibility of specific protein microenvironments, and the cellular location of the protein.

The potential functional significance of regional specificity of β-III tubulin methionine oxidation was considered using the bovine and porcine brain heterodimer models submitted by Nogales and colleagues (Nogales, Wolf et al. 1998; Nogales 1999) to the Protein Data Bank (http://www.rcsb.org/pdb/index.html). Oxidation of β-III tubulin methionines was observed at increased frequency in pathological fractions (SI/FS and NFTs) in regions A63-R77, V155-R162, I163-K175, L263-R276, and A283-K297, which include regions related to GTPase or protein-protein binding functions of β-III tubulin and so have obvious potential functional significance. In addition, the L253-R262 region, which is located adjacent to the MAP binding region, showed methionine oxidation only in pathologic fractions (SI/FS and NFT). L263-R276 is inaccessible in normal protein (de Pereda and Andreu 1996; Nogales, Wolf et al. 1998), suggesting that abnormal protein folding may have preceded increased

methionine oxidation in some regions of β-III tubulin in pathological fractions. Commonly oxidized areas are displayed in Figure 17.

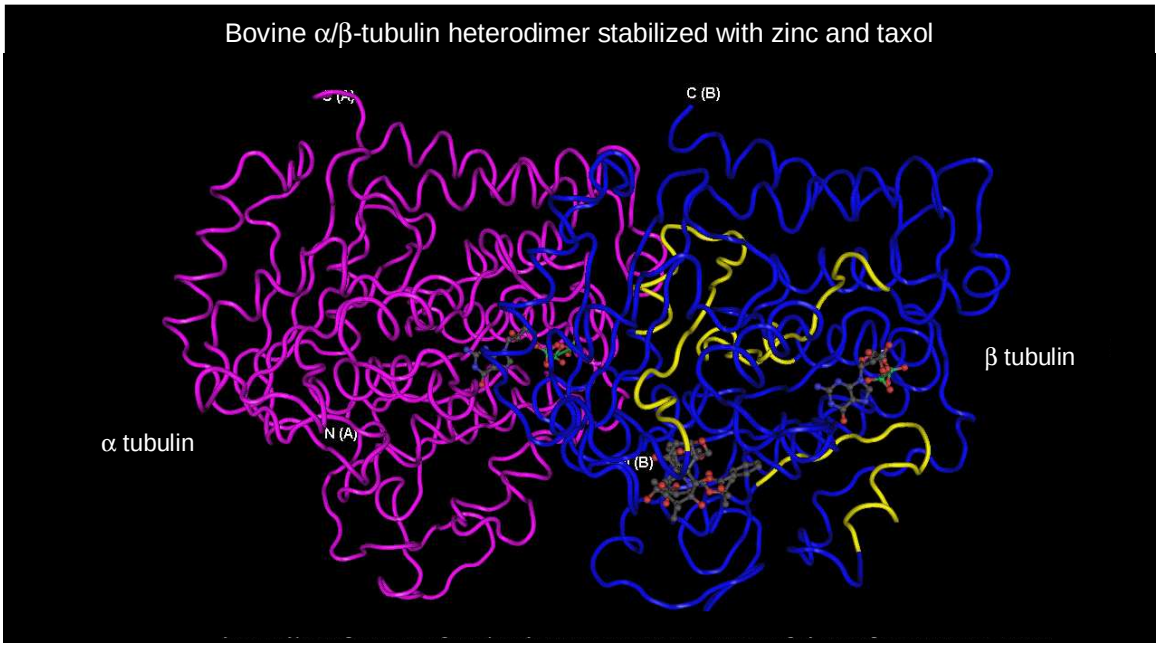


Figure 17. Model of β-tubulin methionine oxidation. Oxidized methionine common to all fractions tested are highlighted in yellow on the β-tubulin (right, colored blue) protein. Structure defines the α/β-tubulin dimer assembled in the presence of taxol and nucleotides in both α and β tubulin subunits.

Some of the LPO adducts mapped in tau were identified within the MT binding regions spanning amino acids from 577 to 683 and therefore have the potential to modify the ability of tau to stabilize MTs (Lee, Neve et al. 1989; Gustke, Trinczek et al. 1994; Perez, Arrasate et al. 2001; Gamblin, Berry et al. 2003). P-MOD-detected mass shifts inferred to be HHE and HNE adducts also were found in the proline-rich region that have been proposed to influence the interaction of tau with MTs (Gustke, Trinczek et al. 1994; Goode, Denis et al. 1997). Prominent N-terminus modification on K24 is in the projection domain of tau, the region that interacts with cellular membranes and other cytoskeletal

elements and is involved in axonal diameter (Chen, Kanai et al. 1992; Brandt, Leger et al. 1995). Among the S, T, and Y residues mapped by P-MOD as containing a +80 amu shift, serines 214, 355, 397, and 400 also have been identified by others using phospho-specific antibodies in pre-tangles and tangles and their phosphorylation is proposed to interfere with tau binding to MTs (Lauckner, Frey et al. 2003). Similarly, P-MOD confirmed tau phosphorylation at S262, T231, and S235, modifications that have been shown to inhibit tau binding to MTs by up to 35% (Sengupta, Kabat et al. 1998).

Finally, it is worth recognizing that although we have identified and mapped oxidative and nitrative modifications to these neuron-enriched cytoskeletal proteins in the detergent-insoluble fraction, other proteins were present in this pathological fraction without an associated increase in oxidative or nitrative modifications. For example, what underlies recruitment of α-III tubulin into the SI/FS fraction is not clear from our studies. One possibility is that α-tubulins are recruited into the detergent-insoluble fraction by virtue of heterodimer formation with modified β-III tubulin; a similar mechanisms has been proposed for apolipoprotein E (Golabek, Kida et al. 2000; Munson, Roher et al. 2000; MacRaild, Stewart et al. 2004; Carter 2005). Still other mechanisms remain. These include protein misfolding and protein modifications that were not a focus of our study and that can lead to changes in function and detergent insolubility (Kato, Nakashima et al. 2001; Diaz-Nido, Wandosell et al. 2002; Munch, Kuhla et al. 2003; Westermann and Weber 2003; Chen, David et al. 2004).

In summary, we have identified, estimated the frequency of, and mapped 12 different mass shifts characteristic of oxidative and nitrative modification on four different cytoskeletal proteins and associated these with three different fractions obtained from AD temporal cortex: normally soluble, detergent insoluble, and presence in LCM NFTs. Our approach was validated by the observation of increased frequency of tau phosphorylation in the pathological fractions. Our results showed selective oxidative modifications of detergent-insoluble β-III tubulin and tau that mapped to functionally important regions of these molecules; independent confirmation of selective modification of β-III tubulin was obtained using CNBr cleavage. Our results suggest that selective oxidative modification of some neuron-enriched cytoskeletal proteins may contribute to protein dysfunction and detergent insolubility that are characteristic of AD pathogenesis.

CHAPTER V

SUMMARY

A well accepted hypothesis of AD etiology is increased Aβ peptide production, aggregation, and deposition. Aβ peptides may directly damage neurons or lead to glial activation, which in turn, leads to increased production and secretion of ROS and RNS. Directly or indirectly, both Aβ and ROS/RNS lead to neuronal LPO and MT collapse, key features of neuronal damage. MT impairment includes detachment and subsequent hyperphosphorylation of tau following alteration of phosphatases and kinases by glial mediated oxidative damage. This project studied how the products of oxidative damage induced aberrant structure of cytoskeletal proteins, a pathological characteristic of AD. The specific aims of this project were to determine if LPO products inhibited the function of the MT, to determine if MT function is similarly inhibited in neurodegenerative diseases, and to determine the type and location of oxidative modifications of cytoskeletal proteins.

The first part of this project studied the effect of exogenous oxidation products on tubulin polymerization. Free radical attack on polyunsaturated fatty acids, such as AA and neuronal DHA, generates intracellular acrolein, HHE, and HNE. In a previous study, N2a cells treated with low concentrations of HNE, a concentration range found and cerebrospinal fluid and brain tissue of AD cases, lead to abnormal morphology of neuronal MTs as assessed by α-tubulin

immunoflourescence compared to untreated cells. To investigate whether morphology and function were both affected, I investigated the alterations of tubulin function by acrolein, HHE and HNE. Pathologically relevant, but subcytotoxic concentrations of LPO, products prevented tubulin polymerization within a concentration range found in AD brain tissue and CSF. Indeed, of the many cytotoxic effects attributed to these LPO products, MT collapse and suppression of tubulin polymerization are among the most sensitive and the most rapid. These data established for the first time that LPO products potently produce irreversible dysfunction of tubulin and suggest a mechanism by which LPO may lead to MT dysfunction in AD.

The second part of this project explored tubulin polymerization within human brain tissue from individuals diagnosed with neurodegenerative disease. Similar to cell culture studies, late onset AD , AD with the presenilin 2 mutation, and frontotemporal dementia with Parkinsonism linked to chromosome 17 had decreased tubulin polymerization in regions heavily affected by NFT pathology. MCI, a prodromal condition that often progresses to LOAD, and age matched control tissue did not have significant decreases in tubulin polymerization, suggesting that tubulin dysfunction, similar to what we observed in cell culture, occurs late in the course of AD pathogenesis. Thus, both cultures of neuronal cells exposed to LPO products and brain tissue from patients with these neurodegenerative diseases had similarly dysfunctional tubulin. What remained unclear was whether or not the tubulin in human neurodegenerative diseases was modified by LPO products, or any form of oxidative damage. We addressed

this gap in our knowledge in the last part of this project.

The final series of studies utilized LC-MS-MS of protein extracts from diseased regions of AD brain and analyzed these spectra with P-MOD. While we observed the expected increase in tau phosphorylation and modification by some LPO products when comparing normal soluble and detergent-insoluble fractions, tubulin showed increased methionine oxidation for β-III tubulin to the exclusion of its binding partner α-III tubulin, a result that we confirmed with biochemical analyses. Some of these modifications to β-III tubulin occurred within regions that are normally hidden from solvent accessible space in a normally folded, fully functional protein. Therefore, a transition from soluble to insoluble tubulin may be related to protein misfolding leaving internal methionines vulnerable to oxidative damage. Protein misfolding is becoming a common theme among neurodegenerative diseases. As far as we are aware, this was the first unbiased characterization of protein modifications associated with transition from soluble to insoluble protein in human disease. Importantly, our results are consistent with the interpretation that methionine oxidation, and not modification by LPO products, may be responsible for the dysfunctional tubulin in AD brain.

Our aged population is poised to expand dramatically within the next decade. In AD pathogenesis studies, the end point hallmarks or lesions are known and well studied; however, the exact processes leading to these lesions are not. Defining early pathological events at the molecular and protein level and targeting appropriate therapies to pre-clinical or early stage dementia is necessary to avert the coming public health crisis. This project showed that LPO

products can lead to MT dysfunction that is characteristic of AD and that this is associated with their accumulation on tau from among the cytoskeletal proteins investigated. In contrast, another type of protein oxidation was observed selectively on β-III tubulin. Together, these data indicate that multiple oxidative modifications to cytoskeletal proteins are likely occurring in AD and that these can contribute to cytoskeletal dysfunction, leading to a modified model of AD pathogenesis (Figure 18). Furthermore, the results suggest that approaches to limit protein oxidation may have the downstream effect of suppressing protein insolubility and its consequences. Perhaps, with further investigation, studies will be able to define drug-treatable targets to prevent and slow neurodegenerative disease progression.

Figure 18.

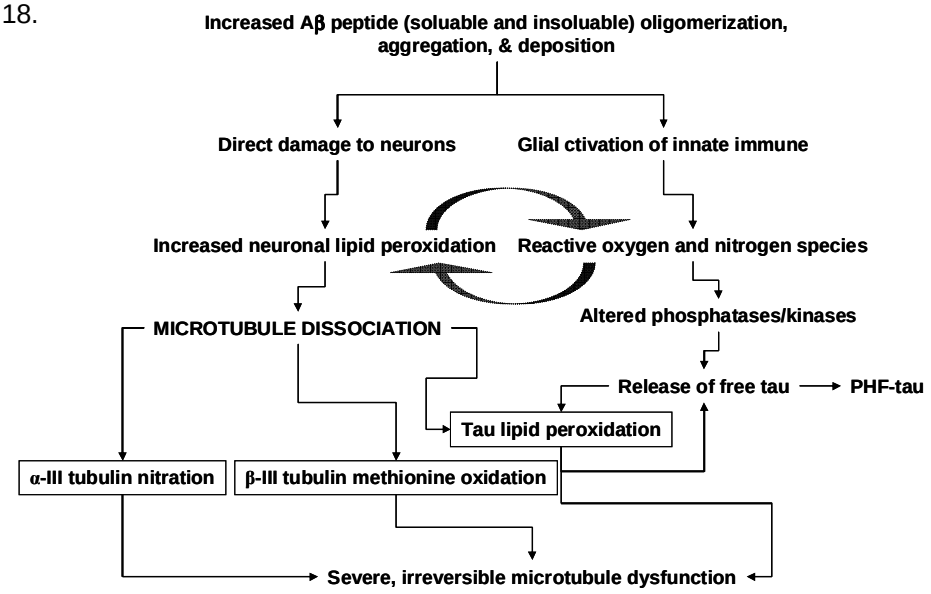


Figure 18. Modified model of AD pathogenesis.

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