Alzheimer’s Disease Pathology as a Clue to Pathogenesis

DISSERTATION

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By

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Abstract

Alzheimer’s disease is characterized pathologically by two intracellular lesions, granulovacuolar degeneration and neurofibrillary tangles. Because Alzheimer’s disease is primarily sporadic, the traditional starting point for studying disease is pathology. It is still debated whether these hallmark lesions are markers or mediators of disease progression; however, the correlation between these lesions and disease progression warrants their use as clues to underlying disease processes. The molecular mechanisms of their development, their role in the disease process, and their connection to one another are not fully understood.

Granulovacuolar degeneration involves the accumulation of large, double membrane-bound bodies within certain neurons during the course of disease. Because of their ultrastructure, it is hypothesized that the bodies are autophagic. To test this, colocalization of autophagic and endocytic markers with a marker for granulovacuolar degeneration was investigated in hippocampal sections prepared from post mortem late stage Alzheimer’s disease cases using double-label confocal fluorescence microscopy. The resultant immunohistochemical signature suggests that granulovacuolar degeneration bodies accumulate at the nexus of autophagic and endocytic pathways and that failure to complete autolysosome formation may correlate with their formation. Due to its far-
reaching roles in cells, disruption of the endocytic pathways has the potential to act as a nidus central to disease onset and the development of granulovacuolar degeneration as well as neurofibrillary tangles and amyloid plaques.

In sporadic Alzheimer’s disease, neurofibrillary tangle formation is preceded by extensive post-translational modification of tau. To identify the modification signature associated with tau lesion formation at single amino acid resolution, paired helical filaments immunopurified from Alzheimer’s disease brain were subjected to liquid chromatography, tandem mass spectrometry analysis. The resulting spectra identified Lys monomethylation as a new tau modification distributed among seven residues located in the projection and microtubule binding domains of tau protein. One site, K254, was found to be a substrate for a competing Lys modification, ubiquitylation. Double label confocal fluorescence microscopy demonstrated that methylation is wide-spread among neurofibrillary tangles in hippocampal sections prepared from post mortem late-stage Alzheimer’s disease cases. Together these data provide the first evidence that tau in neurofibrillary lesions is modified by Lys methylation.

The tau methylation signature involves sites known to mediate disease-related modifications including ubiquitylation and phosphorylation, suggesting that methylation is a candidate modification for influencing tau aggregation, toxicity, and protein turnover. To extend the correlation between methylation occupancy and disease, the modification state of soluble tau protein isolated from cognitively normal human brain was investigated using proteomic methods. Results showed that normal soluble tau is hypermethylated relative to disease-derived tau in its microtubule binding domain.
Furthermore, recombinant human tau subjected to reductive methylation *in vitro* retains the normal function of tau as a microtubule-binding and stabilizing protein but its aggregation propensity is greatly attenuated. These data establish Lys methylation as a normal tau post-translational modification in human brain that may protect against pathological tau aggregation during aging. Furthermore, data suggests that enzymes responsible for tau methylation, including methyltransferases and demethylases, may be tractable targets for disease-modifying therapies focused on halting neurofibrillary lesion formation in Alzheimer’s disease.
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2N4R, two N-terminal region four microtubule binding repeats, full length tau; Aβ, Amyloid beta; AD, Alzheimer’s disease; APP, Amyloid precursor protein; BACE, Beta secretase; CHMP2B, Charged multivesicular body protein 2B; Ckiδ, Casein kinase 1 delta; CSF, Cerebrospinal fluid; CTSD, Cathepsin D; e2, e3, e10, Protein sequence encoded by exons 2, 3, 10, respectively; ESCRT, Endosomal sorting complex required for transport; GVB, Granulovacuolar degeneration body; GVD, Granulovacuolar degeneration; LAMP1, Lysosome-associated membrane protein 1; LC3, Microtubule-associated protein 1 light chain 3; LC-MS/MS, Liquid chromatography, tandem mass spectrometry; MAPT, microtubule associated protein tau; me, Methyl; MTBR, Microtubule binding region; MVB, Multivesicular body; NFT, Neurofibrillary tangle; PB, Phosphate buffer; PHF, Paired helical filament; PMI, Post mortem interval; PP2A, Protein phosphatase 2A; PS1, Presenilin 1; p-tau, Phosphorylated tau; PTM, Post-translational modification; SAM, S-adenosylmethionine; TEM, transmission electron microscopy; ub, Ubiquitin
Chapter 1: Introduction

1.1. Alzheimer’s disease pathology

Alzheimer’s disease (AD) is defined by the appearance of pathological hallmarks within specific areas of the brain, including amyloid plaques, composed of extracellular amyloid beta (Aβ) peptide (Selkoe, 1998), and neurofibrillary tangles (NFTs), composed of intracellular aggregates of the microtubule-associated protein tau (Goedert, 1993). Definitive diagnosis of AD is made at autopsy on the basis of these defining lesions, which have a well documented but poorly understood connection to disease. Although it has been debated whether these lesions are mediators of disease, they have clear correlation to disease progression and are useful as clues to understanding the pathological processes accompanying neurodegeneration. Additional hallmarks of AD include granulovacuolar degeneration (GVD), characterized morphologically as intracellular double membrane-bound organelles harboring a dense core, and extracellular tau detectable in the cerebrospinal fluid (CSF) (Ball and Lo, 1977; Holtzman, 2011). Although the molecular mechanisms for individual pathological lesions have been proposed (Goate et al., 1991; Smith et al., 1996; Hasegawa, 2007), a unifying hypothesis that rationalizes the appearance of all hallmarks of disease has been elusive. Here we review recent studies involving the intracellular vesicular pathways and their connection
to the hallmark lesions of AD. We propose that endocytic pathway dysfunction, and more specifically a failure in lysosomal fusion, may be the nidus of both defining lesions of AD as well as of GVD and extracellular tau, thus providing a unifying hypothesis for disease pathology.

1.2. Vesicle Trafficking Dysfunction

Intracellular vesicle trafficking pathways form an interconnected, dynamic system for transfer of cellular constituents within the cell and between intracellular and extracellular compartments (Mellman, 1996) (Figure 1.1). Cell surface proteins can enter the system through endocytosis. For example, the epidermal growth factor receptor is endocytosed and incorporated into early endosomes after binding its ligand and becoming ubiquitylated (Vieira et al., 1996; Vanlandingham and Ceresa, 2009). Endocytosed material can then be budded off into recycling endosomes for exocytic release or incorporated into intralumenal vesicles of the multivesicular body (MVB) by the sequential activity of the endosomal sorting complexes required for transport (ESCRTs) (Saksena et al., 2007; Williams and Urbe, 2007; Hurley, 2008). Intralumenal vesicles and their contents may also be released as exosomes by fusion of the outer membrane of the MVB with the plasma membrane (Johnstone et al., 1991; Thery et al., 2002). The endocytic pathway merges with the macroautophagic (hereafter referred to as simply autophagic) pathway at either the early endosome or MVB stage (Fader and Colombo, 2009). In the autophagic pathway, target proteins are polyubiquitylated, fostering the binding of p62, the autophagic protein microtubule-associated protein 1
light chain 3 (LC3) (Pankiv et al., 2007), and the assembly of the autophagic membrane around the ubiquitylated cargo to form the autophagosome (Kabeya et al., 2000). The autophagosome can then fuse with the lysosome directly or with the MVB to form the amphisome, a slightly acidic hybrid organelle. The amphisome also can fuse with the lysosome to form the autolysosome, a caustic organelle that degrades the enclosed proteins and organelles (Dunn, 1990; Eskelinen, 2005).

The dynamic nature of this system is difficult to capture in fixed post mortem tissue, however there are several lines of evidence suggesting that changes in pathway flux are among the earliest pathologies observed in AD, preceding clinical symptoms of AD, intracellular NFT formation, and extracellular amyloid deposition (Nixon et al., 2001; Nixon and Cataldo, 2006). First, expression profiling during the progression of AD has revealed significant upregulation of effector genes of the early endosome (including rab4 and rab5), the late endosome (rab7), and the exocytic pathway (rab27) (Ginsberg et al., 2010a; Ginsberg et al., 2010b; Ginsberg et al., 2011). These expression changes are consistent with morphological phenotypes observed in AD. For example, overexpression of rab5 causes enlarged endosomes, one of the earliest pathological alterations observed in AD (Cataldo et al., 2008). Rab7 upregulation is found in vulnerable hippocampal basal forebrain regions, but not in relatively spared striatum and cerebellum in mild cognitive impairment and AD (Cataldo et al., 2000; Ginsberg et al., 2010b). Second, the accumulation of Aβ and tau protein and the appearance of GVD bodies correlate with changes in trafficking pathways. These data are summarized below.
1.2.1. Aβ Accumulation

Observations in both human tissue and cell culture implicate the endocytic pathway in Aβ production (Cataldo et al., 1997; Nixon et al., 2000; Nixon et al., 2001; Mathews et al., 2002; Grbovic et al., 2003; Cataldo et al., 2004a). AD-related endocytic dysfunction coincides with the detection of Aβ within endosomal compartments and autophagic vacuoles that collect within dystrophic neurites with the initial rise in soluble Aβ peptides (Mathews et al., 2002; Grbovic et al., 2003; Yu et al., 2005). The presence of Aβ in early endosomes also is consistent with the colocalization of APP and BACE1 within the same early endocytic compartments (Huse et al., 2000; Kametaka et al., 2003; Kinoshita et al., 2003; Arbel et al., 2005) and the degradation of BACE within the endosomal-lysosomal system (Koh et al., 2005). One study suggests that internalized Aβ can aggregate within the cell and disrupt the vesicular membrane, thus contributing to its pathologic effect (Friedrich et al., 2010). Intracellular trafficking of proteins involves a series of cytosolic factors, some of which are implicated in the regulation of APP trafficking and Aβ generation. For example, Rab6, a protein implicated in membrane budding, and clathrin, which mediates the internalization of APP from the cell surface, affect APP processing (Koo and Squazzo, 1994; McConlogue et al., 1996). Altogether, these data support a relationship between endocytic pathway dysfunction and the amyloidogenic processing of APP.

Lysosome-related pathology also is greatly accentuated in familial AD, most commonly caused by presenilin 1 (PS1) mutations (Cataldo et al., 2004b). PS1 is a transmembrane protein that acts as the catalytic subunit of the γ-secretase complex
(Kimberly et al., 2003; Takasugi et al., 2003). This complex is located in the endoplasmic reticulum, transgolgi network, and endocytic compartments (Kovacs et al., 1996; Cupers et al., 2001). Familial AD-linked PS1 mutations significantly reduce budding from the endoplasmic reticulum and golgi and result in decreased delivery of APP to the cell surface (Cai et al., 2003). This suggests that familial AD-linked PS1 variants increase Aβ production by decreasing intracellular transport of APP, thus prolonging the availability of APP for cleavage by β- and γ-secretases within the golgi. PS1 may regulate protein trafficking through its interaction with several cytosolic factors involved in the regulation of vesicular transport, such as Rab11, Rab6 and Rab regulators (Dumanchin et al., 1999; Scheper et al., 2000; Scheper et al., 2004). Other data has ascribed the pathogenic effects of PS1 mutations to its role in facilitating maturation and targeting of a subunit of the vacuolar ATPase to the lysosome, which is essential for lysosomal acidification, protease activation, and degradation of lysosomal substrates (Lee et al., 2010). This corroborates the impaired lysosomal fusion hypothesis in that Bafilomycin A, a vacuolar ATPase inhibitor, is known to impair fusion of the lysosome with other membrane bound vesicles (Yamamoto et al., 1998). Because Aβ is both generated and degraded via the endocytic and autophagic pathways (Yu et al., 2005), impaired lysosomal clearance could account for PS1-mediated increases in Aβ. Furthermore, the commonalities between sporadic and familial AD endocytic pathology suggests that lysosomal dysfunction may be the nidus of AD pathogenesis.
1.2.2. Tau

1.2.2.1. Extracellular Tau.

Tau and phospho-tau (p-tau) appear in CSF (Holtzman, 2011). It has been assumed that this observation results from the passive release of tau from dying neurons. However, recently it was shown that tau may be actively secreted into the extracellular compartment, where it is positioned to participate in the transmission of neurofibrillary pathology (Kim et al., 2010a; Kim et al., 2010b). Exosome-mediated release is considered a common, yet unconventional, mechanism responsible for the secretion of other aggregation-prone proteins including α-synuclein (Emmanouilidou et al., 2010), prion protein (Fevrier et al., 2004), Aβ (Rajendran et al., 2006), and tau (Saman et al., 2012). Secreted tau associates with both typical exosomal proteins, such as Alix, and with proteins involved in tau misprocessing and AD pathogenesis, such as Aβ, presenillins, and fyn kinase (Saman et al., 2012). Furthermore, exosomal tau is phosphorylated at Thr-181, accordant with CSF data (Saman et al., 2012). Somewhat surprisingly, the greatest amount of exosomal tau was found in early AD, with the greatest being in Braak stage 3, progressively less so in later stages of AD when neuronal death is rampant, and absent from non-AD controls diagnosed with non-tauopathic dementias (Saman et al., 2012). Because there is relatively little neurofibrillary degeneration at this early stage (Braak et al., 2006), the elevation of p-tau in AD CSF is not due to passive, nonspecific tau release consequent to neuronal death. Rather, these data highlight the vesicle trafficking pathway involved in tau processing and secretion.
A lysosomal fusion defect may account for the disease-related detection of tau in the CSF. The p-tau that accumulates where dendritic microtubules are being lost in dendrites is largely vesicle-associated, and some of these vesicles are amphisome-like, harboring both endocytic (fyn) and autophagic (LC3) markers (Lee et al., 2012). Fyn activation during signal transduction typically causes the oligomerization and endocytosis of downstream elements (Sverdlov et al., 2007) and targets at least some oligomerized targets of fyn phosphorylation to exosomes (Nickel, 2005; Fang et al., 2007). Although in normal autophagy, LC3 is rarely exocytosed (Dolan and Johnson, 2010), the inability of the autophagic vesicle to fuse with the lysosome may alternatively shuttle vesicles towards the exosomal pathway (Figure 1.1). This exosomal secretion of tau corroborates the recent suggestion that interneuronal transfer of tau may be an important aspect of pathogenesis and account for the stereotypic neurofibrillary lesion progression (Braak and Braak, 1991; Armstrong et al., 1998; Armstrong et al., 1999; Gomez-Ramos et al., 2006; Gomez-Ramos et al., 2008). Furthermore, this is consistent with recent evidence gathered from the lysosomal storage disorder, Niemann-Pick type C disease, in which exosomal release of cholesterol may serve as a cellular mechanism to partially bypass the traffic block that results in its toxic accumulation within the lysosome (Strauss et al., 2010). Altogether, although tau lacks the usual features of secreted proteins, such as an N-terminal hydrophobic “leader” sequence and lipidation sites, data suggests that tau is secreted, though it may be low in healthy neurons, such as when the lysosomal system is functioning normally (Kim et al., 2010b).
1.2.2.2. Intracellular Tau.

Failure of the lysosome to efficiently fuse with membrane-bound organelles and degrade encaptured proteins of the endocytic and autophagic pathways may also directly affect the intracellular concentrations of proteins, including tau. Two major proteolytic systems contribute to protein degradation in cells: the ubiquitin-proteosome system and the autophagy-lysosome system. There is putative evidence both supporting (David et al., 2002; Zhang et al., 2005) and opposing (Brown et al., 2005; Delobel et al., 2005; Feuillette et al., 2005) tau degradation via the proteasome. Alternatively, there is evidence to suggest that tau can be degraded by lysosomal proteases such as cathepsin D in vitro and in the cytosol (Bednarski and Lynch, 1996; Kenessey et al., 1997), and that cathepsin B closely associates with intracellular neurofibrillar tangles in AD brains (Ii et al., 1993). Furthermore, inhibition of lysosomes can increase tau levels (Bendiske and Bahr, 2003), and tau was found in neuronal lysosomes of both AD and control brain (Ikeda et al., 1998). Also, abnormal tau-actin interactions (Fulga et al., 2007) and the misrouting of tau to the golgi (Farah et al., 2006) in tauopathy suggest that an abnormal association with the plasma membrane and/or trafficking vesicles derived from it may also play a role in tau-mediated toxicity. Moreover, inhibition of autophagy not only depressed the degradation of tau aggregates but also accelerated tau aggregation and cytotoxicity (Wang et al., 2009). It is interesting to note that tau also encodes two motifs for chaperone-mediated autophagy (Wang et al., 2009), a form of autophagy selective for soluble cytosolic proteins that relies on hsc70 to target substrates to the lysosome. However, it is unclear whether tau is degraded via chaperone mediated autophagy in vivo.
Thus failure of the lysosomal pathway to efficiently clear intracellular tau may foster its pathological aggregation as well as its pathogenic release in exosomes.

Similar endocytic abnormalities may be involved in non-AD tauopathies, independent from Aβ deposition. Tau is redistributed to microtubule-poor regions of the cell when it is present in excess of available microtubule binding capacity, which can result from overexpression, mutation, or post-translational modifications (PTMs) that limit tau-microtubule binding (Samsonov et al., 2004; Lee et al., 2012). Since tau has been shown to modulate the activity of microtubule-associated motor proteins that mediate dendritic transport (Dixit et al., 2008; Kapitein et al., 2010), it is possible that toxicity resulting from tau accumulation at localized dendritic loci may have relevance to pathogenesis of non-AD tauopathies. Moreover, this toxic, vesicle-associated tau accumulates selectively in microtubule-poor segments containing organized microtubule bundles, suggesting that its accumulation is both the cause and consequence of localized microtubule destabilization (Lee et al., 2012). Thus, even in the absence of Aβ deposits, alterations in tau may accelerate endocytic dysfunction, which then in turn, may accelerate NFT formation.

1.2.2.3. Mechanisms of Tau Regulation.

The mechanisms regulating tau secretion through the intracellular vesicular trafficking pathways are unknown but may involve those also responsible for modulating pathological and normal tau biology, including alternative splicing and PTMs. Tau protein is encoded by a single gene comprising 16 exons (Neve et al., 1986; Almos et al.,
Exons 2, 3 and 10 undergo alternative splicing; however exon 3 is expressed only in the presence of exon 2, thus yielding 6 isoforms. Depending on the presence or absence of the protein sequence encoded by exon 10 (e10), tau isoforms are called 4R (with e10) or 3R (without e10), referring to the number of imperfect microtubule binding repeats. Similarly, tau isoforms are called 0N (without N-terminal inserts), 1N (with one N-terminal insert, encoded by exon 2), or 2N (with two N-terminal inserts, encoded by exons 2 and 3). E10 has received relatively more attention than either N-terminal inserts (e2 and e3) due to its role in microtubule binding and self-aggregation. However, recent evidence suggests that while extracellular secretion of tau requires the presence of an unknown element in the N-terminal domain (Kim et al., 2010b), e2 specifically inhibits this secretion (Kim et al., 2010a). This agrees with known functions of the N-terminus of tau to mediate association with the plasma membrane and perimembranous structures (Brandt et al., 1995).

In conjunction with the inclusion or exclusion of e10, the affinity of tau for microtubules is regulated by its phosphorylation at sites in and around the microtubule binding repeat region, with certain sites having more acute affects on the protein than others (Abraha et al., 2000; Haase et al., 2004; Liu et al., 2007). Exosomal fractions of conditioned media of human neuroblastoma cells are enriched in p-tau species associated with neurodegeneration (Saman et al., 2012). Quantitative analysis has identified four epitopes within the proline-rich domain of tau that are most strongly enriched in secreted tau compared with intracellular tau (Saman et al., 2012). These epitopes include AT270, AT8, AT100, and AT180, which correspond to phosphorylation at T181, S198 and S202,
S210 and T212, and T231, respectively. Significantly, epitope AT270 (corresponding to phosphorylated T181) is the epitope most highly enriched in secreted tau and is also an established biomarker for CSF-based diagnostics for early-stage AD (Vanderstichele et al., 2006; Saman et al., 2012). The potential role of phosphorylation as a regulator of tau secretion is corroborated by CSF biomarker data gathered from patients with acute brain injury, which at the cellular level involves axonal injury (Smith et al., 2003). Importantly, total tau, but not p-tau, increases following acute brain injury (Holtzman, 2011), suggesting that while total tau may be released as a result of cellular damage and death, PTMs may actively regulate tau secretion.

As many as 30 different phosphorylation sites have been identified on tau (Buee et al., 2000), and although phosphorylation is the most comprehensively studied of the tau PTMs, filamentous tau is known to be extensively modified by several PTMs, including Lys-directed ubiquitylation (Martin et al., 2011). We and others have identified at least three sites of ubiquitylation on tau (Morishima-Kawashima et al., 1993; Cripps et al., 2006; Thomas et al., 2012). Although most well known for its role in proteasomal degradation of proteins, ubiquitin is also the best known signal for endocytic sorting (Raiborg et al., 2003). As a general rule, targeting to the proteasome requires attachment of a chain of at least four ubiquitins (Chau et al., 1989), whereas targeting to the endocytic pathway requires only a single ubiquitin or a short chain of two or three ubiquitins (Raiborg et al., 2003). These ubiquitins are recognized by the ESCRT machinery, which sort the ubiquitylated cargo and direct it towards its destined pathway (Saksena et al., 2007; Williams and Urbe, 2007). In addition to the influence that PTMs
exert on tau function individually, it is thought that various modifications may cooperate and compete in a coordinated fashion. For example, recently two modifications have been identified that are also Lys-directed: acetylation (Min et al., 2010; Cohen et al., 2011) and methylation (Thomas et al., 2012). Because these modifications both directly compete with ubiquitin for Lys site occupancy, there is potential for acetylation and methylation to directly affect the rate of turnover of tau protein and its motility through the endocytic pathway. Furthermore in other biochemical pathways, such as histone-regulated processes, there is precedent for PTMs, including acetylation and methylation, to indirectly affect neighboring site modification, such as phosphorylation (Latham and Dent, 2007), though the potential for this type of modification crosstalk to be occurring in tau protein and its effect on tau metabolism has yet to be examined.

1.2.3. GVD Bodies

Evidence of dysfunctional endocytic/autophagic pathway in AD extends to GVD. GVD body load increases with disease severity and episodic memory decline (Ball, 1977; Ball and Lo, 1977; Ghoshal et al., 2002). GVD body ultrastructure has been extensively studied, revealing an electron-dense core with coarse or vesicular morphology surrounded by a double-layered membrane (Okamoto et al., 1991). Because of the two-layered membrane, it has long been hypothesized that these lesions are of autophagic origin (Okamoto et al., 1991). Recent work by us and others has implicated the endocytic pathway in the formation of GVD bodies due to strong immunoreactivity of CHMP2B, a component of ESCRT-III, in the GVD body core (Yamazaki et al., 2010; Funk et al.,
This work also suggests a failure of the MVB to fuse with the lysosome (Funk et al., 2011), which could account for an accumulation of autophagic intermediates (Boland et al., 2008) and an increased size and density of the MVB (Vanlandingham and Ceresa, 2009). We hypothesize that consequently, flux is routed to the autophagic pathway, which results in the accumulation of abnormally large amphisome-like intermediates owing to the same lysosomal fusion defect combined with mTOR-mediated suppression of phagophore formation (Chen et al., 2005; Diaz-Troya et al., 2008; Soliman et al., 2010). The genetic increase in rab7 in AD (Ginsberg et al., 2010a; Ginsberg et al., 2011) is somewhat inconsistent with this hypothetical lysosomal fusion failure, though it is possible that this upregulation is acting in an ineffective compensatory manner. However, it is interesting that late endosome marker rab24, but not secretory protein rab27, is upregulated in CA1 pyramidal neurons (Ginsberg et al., 2010a; Ginsberg et al., 2011) considering that GVD most greatly affects the CA1 region of the hippocampus (Ball and Lo, 1977). This may reflect alternative responses to the disease process with one cell type opting for increased secretion and another for increased degradation to remove the toxic protein.

1.3. Conclusions

We propose that lysosomal fusion dysfunction is a candidate nidus for the major pathological hallmarks of AD including both defining lesions of AD, GVD bodies, as well as the presence of tau in the CSF. The involvement of lysosomal dysfunction as an underlying and unifying hypothesis for AD pathology may change the overall view of
AD pathogenesis. It provides novel insights into disease-associated mechanisms of protein misprocessing and potentially new modes of disease progression. There is also potential clinical importance of tau secretion biomarkers for CSF-based diagnostics and for the direction of future disease-modifying therapeutics.
1.4. Figures

Figure 1.1. Intracellular vesicular trafficking pathway.
Endocytosed surface proteins, such as APP, are delivered to the endosomal system by internalization. Internalized membrane proteins can be sorted into intraluminal vesicles of the MVB by sequential activity of ESCRTs 0, I, II, and III, or delivered to the extracellular environment by the recycling endosome. Mature MVBs fuse with either the autophagosome of the autophagic pathway to form the amphisome or directly with the lysosome, which donates degradative hydrolases, creating the autolysosome where complete degradation of the sequestered material occurs. Alternatively, the multivesicular body can fuse with the plasma membrane, resulting in the exosomal secretion of the intralumenal vesicles and their internalized cargo.
Chapter 2: Granulovacuolar Degeneration Bodies of Alzheimer’s Disease Resemble Late-stage Autophagic Organelles

2.1. Introduction

Granulovacuolar degeneration (GVD) is characterized by the intraneuronal accumulation of large (up to 5 μm diameter) membrane-bound vacuoles harboring a central granule. Although originally observed in Alzheimer’s disease (AD) (Simchowicz, 1911), and classically detected in hippocampal pyramidal neurons (Ball, 1978), GVD also affects additional brain regions (Xu et al., 1992; Yamazaki et al., 2010) and is found above age-matched control levels in other adult-onset dementias including forms of frontotemporal lobar degeneration (Lagalwar et al., 2007). In AD hippocampus, granulovacuolar degeneration body (GVB) load increases with disease severity in parallel with neurofibrillary lesion density (Ball, 1977; Ball and Lo, 1977) and correlates strongly with decline of episodic memory performance (Ghoshal et al., 2002). Much like the defining lesions of AD, β-amyloid plaques and neurofibrillary tangles, GVBs have a well-documented but poorly understood connection to disease progression. Elucidation of GVB composition and origin may offer additional insight into the pathogenesis of sporadic disease.
Toward that end, GVB ultrastructure has been extensively studied, revealing a central electron-dense granule with coarse or vesicular morphology and a surrounding double unit membrane (Okamoto et al., 1991). On the basis of the latter characteristic, a macroautophagic origin for GVBs has been proposed (Okamoto et al., 1991). Macroautophagy (hereafter referred to as autophagy) begins with the formation and extension of an isolation membrane, or phagophore, that is double-layered and decorated by the autophagic membrane protein LC3 (microtubule-associated protein 1 light chain 3; Figure 2.1) (Arstila and Trump, 1968; Kabeya et al., 2000; Kabeya et al., 2004). Upon closure of the membrane, the resultant autophagosome undergoes a stepwise maturation process involving fusion with late endosomes/multivesicular bodies to form amphisomes, and finally with lysosomes to yield autolysosomes (Figure 2.1). The latter maturation steps acquire ATPases that acidify the vacuolar lumen, and lysosomal hydrolases such as cathepsins that degrade the inner autophagic membrane and its sequestered cargoes (Dunn, 1990; Eskelinen, 2005). Digested materials are then transported back to the cytoplasm and recycled (Fader and Colombo, 2009). Because autophagy is a dynamic and efficient process in healthy neurons, autophagic intermediates are rarely observed unless their formation or clearance is inhibited (Boland et al., 2008). Whether autophagic misfunction contributes to GVB formation is unknown.

Although autophagy is traditionally considered a non-specific process for bulk degradation of cytosolic components, it also mediates selective turnover of polyubiquitinated misfolded proteins that accumulate in disease and cellular organelles (Kaniuk et al., 2007; Kim et al., 2008a). Consistent with this potential function, GVB
granules contain ubiquitin immunoreactivity (Okamoto et al., 1991; Lübke et al., 1993). On the other hand, neurons undergoing GVD only modestly overlap with those containing tau aggregates (Holzer et al., 2002; Kannanayakal et al., 2006), and GVB granules contain only weak phospho-tau immunoreactivity (Dickson et al., 1987; Bondareff et al., 1991; Mena et al., 1992; Ikegami et al., 1996). Moreover, neither induction nor inhibition of autophagy alone has been reported to yield GVBs in model systems (Boland et al., 2008). Autophagy is but one process through which cellular constituents enter lysosomes for degradation (Figure 2.1), and more than one pathway may contribute to the formation of GVBs.

Traditionally, GVD has been detected with non-specific histological stains such hematoxylin and eosin, which has slowed progress in characterizing GVB molecular composition. However, we reported that Ckiδ, a protein kinase implicated in protein ubiquitination, is a robust surrogate marker for the GVB granule (Ghoshal et al., 1999). Through this approach it is now possible to conduct GVB colocalization experiments in authentic AD tissue. Here we use immunohistochemistry to probe the relationship between GVBs and representative proteins associated with the autophagic and endocytic processes. The results show that GVBs contain autophagic and endocytic markers, and have an epitope signature most consistent with the amphisome stage of autophagy. The results point to incomplete lysosome fusion as a potentially important correlate of GVB formation.
2.2. Materials and Methods

2.2.1. Subjects

Postmortem brain tissue was obtained from five elderly subjects with a clinical diagnosis of AD (mean age ± SD of 71 ± 11 years; Table 2.1) that was confirmed on neuropathological evaluation in which the Consortium to Establish a Registry for AD (CERAD) age-adjusted criteria were met (Mirra et al., 1991). All cases satisfied criteria for Braak stages IV or V (Braak and Braak, 1991).

2.2.2. Tissue preparation and Immunohistochemistry

Hippocampal brain tissue obtained postmortem was fixed in either methacarn (methanol:chloroform:acetic acid; 6:3:1) or phosphate-buffered 20% formalin (8% formaldehyde), embedded in paraffin, and sliced into 6-μm thick sections. Tissue sections were then deparaffinized in xylene, rehydrated in a descending series of ethanol concentrations, then subjected to antigen retrieval (low microwave heating for 20 min; (Evers et al., 1998)) in citrate buffer (10 mM citric acid, 0.5% Tween-20, pH 6.0) followed by washing (2 x 5 min) in PBST (2.7 mM KCl, 0.14 M NaCl, 8.1 mM Na₂HPO₄, 0.1% Tween-20, pH 7.4). Washed sections were then blocked (1 h at 19°C) with 5% goat serum diluted in phosphate buffer (PB; 33 mM NaH₂PO₄, 162 mM Na₂HPO₄, pH 7.4), then incubated (overnight at 4°C) with primary antibodies (listed in Table 2) diluted in PB containing 2.5% goat serum. After washing in PBST (3 x 10 min), sections were incubated (1 h at 19°C) with fluorescent dye-labeled secondary antibodies (1.5 μg/ml Cy3-conjugated goat anti-rabbit IgG, Jackson Immuno Research Laboratories,
Inc, West Grove, PA; 2 μg/ml Alexa Fluor 488-conjugated goat anti-mouse IgG, Invitrogen, CA). After washing in PBST (3 x 10 min), tissue was treated (10 min at 19°C) with 0.1% Sudan Black B (EM Diagnostics, Gibbstown, NJ) in 80% ethanol to suppress lipofuscin autofluorescence (Schnell et al., 1999). Sections were then washed (2 x 10 min) in PBST and once for 10 min in PB. Coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and sealed with clear nail enamel. Labeled sections were viewed in a Leica TCS SL laser-scanning confocal system (100x oil HCX Plan Apo CS 0.70 - 1.40 NA objective lens) operated at wavelengths optimized for simultaneous detection of Alexa Fluor 488 (λ<sub>ex</sub> = 488 nm; λ<sub>em</sub> = 500-530 nm) and Cy3 (λ<sub>ex</sub> = 543 nm; λ<sub>em</sub> = 560-600 nm). Long wavelength fluorescence (λ<sub>ex</sub> = 633 nm; λ<sub>em</sub> = 650-740 nm) also was monitored to assess autofluorescence intensity. Digital confocal images were captured at 1x and 4x digital zoom, and stored in Tagged Image Format. Both secondary antibodies displayed minimal non-specific staining under these conditions, as determined by immunostaining in the absence of primary antibodies.

2.2.3. Assessment of colocalization of GVD with autophagy markers

To assess the relationship between autophagy and GVD in AD hippocampus (Braak stages IV and V, Table 1), the distribution of various marker proteins (Table 2) was compared to that of GVBs (marked by Ckiδ) using double-label confocal immunofluorescence microscopy. Colocalization was assessed both at the cellular and subcellular levels, and was limited to only those GVBs having a diameter of at least 1 μm to facilitate quantification. At the subcellular level, colocalization was defined as
“granular” when marker immunoreactivity directly overlapped Ckiδ immunoreactivity in a GVB granule, and as “encircling” when it surrounded the periphery of a GVB vacuole. The two types of subcellular colocalization, encircling and granular, were pooled from multiple observations from each case (technical replicates) and quantified as the mean percent colocalization. At the cellular level, colocalization was defined as the presence of at least one example of the encircling or granular patterns in a neuron undergoing GVD. For both cellular and subcellular observations, overall mean percent colocalization was determined from the average of all five cases and was expressed ± standard error of the mean.

2.2.4. Analytical evaluation of immunohistochemical staining and statistics

The proportion of each marker colocalizing with Ckiδ-immunoreactive GVBs containing granules at least 1 µm in diameter was estimated for each case as a mean ± 95% confidence interval using the Wilson score method (Newcombe, 1998). Sufficient technical replicates were counted from at least 10 fields of each case so that the 95% confidence interval was not more than 15%. Overall mean colocalization was then calculated as the average of all five biological replicate means and reported ± standard error of the mean. Analysis was conducted at both the cellular (colocalization with neurons undergoing GVD) and subcellular (colocalization with individual GVBs) levels.

Differences in colocalization of markers with GVBs were assessed by z-test:

\[ z = \frac{x_1 - x_2}{\sqrt{(s_1^2 + s_2^2)}} \]  

(Eq. 2.1)
where \( x_1 \pm S_{x_1} \) and \( x_2 \pm S_{x_2} \) are the pair of overall mean colocalization estimates ± SE being compared, \( z \) is the 1-\( \alpha \) point of the standard normal distribution, and \( p \) (the probability of obtaining the observed results assuming the null hypothesis) is 2\( \alpha \). All statistical analyses were carried out using JMP 8 (SAS Institute, Cary, NC).

2.3. Results

2.3.1. Colocalization with early-stage autophagy markers

Early-stage autophagy was probed with antibodies raised against markers LC3 and p62 (Table 2.2). The lipidated form of LC3 (LC3-II) is bound by both the inner and outer membrane of the autophagosome, and thus colocalizes with the earliest stages of autophagosome formation (Figure 2.1) (Kabeya et al., 2000; Mizushima, 2004). LC3-II decreases with autophagosome maturation, however, owing to partial proteolysis and delipidation of the outer-membrane by cysteine protease Atg4 (Kimura et al., 2007) and destruction of the inner membrane by lysosomal/endosomal hydrolases. Therefore, LC3 primarily marks the phagophore and autophagosome relative to late-stage compartments (i.e., the amphisome and the autolysosome). When AD hippocampus was investigated by double-label immunofluorescence microscopy (Figure 2.2), Ckiδ immunoreactivity displayed the punctate cytoplasmic pattern previously shown to correspond to GVBs (Ghoshal et al., 1999). In contrast, LC3 immunoreactivity was typically diffuse and filled neuronal cell bodies (Figure 2.2) in agreement with a previous report (Ma et al., 2010). However, the diffuse LC3 staining pattern did not colocalize with GVBs at the
cellular or subcellular levels (**Figure 2.3**), suggesting that GVBs do not correspond to early-stage autophagy organelles.

To confirm this finding, the distribution of p62 was investigated. p62 binds ubiquitin, and is commonly found associated with polyubiquitinated protein aggregates including those in neurofibrillary tangles (Kuusisto *et al.*, 2001; Kuusisto *et al.*, 2002; Kuusisto *et al.*, 2008). It also binds LC3, and so can act as an adaptor protein to facilitate autophagic degradation of ubiquitinated substrates (Pankiv *et al.*, 2007). Thus, p62 immunoreactivity predominates in cells containing ubiquitinated protein aggregates and accumulates in non-degradative autophagic compartments (phagophore and autophagosome) relative to amphisomes and autolysosomes where it is destroyed. Double-label immunofluorescence microscopy confirmed that p62 colocalized with neurofibrillary tangles, however it rarely colocalized with GVBs at the cellular or subcellular levels (**Figure 2.2g-l; Figure 2.3**). This low level of colocalization did not differ significantly (at $p < 0.05$) from that observed for LC3 at the cellular (**Table 2.3**) or subcellular (**Table 2.4**) levels. Together, both LC3 and p62 immunostaining patterns suggest that GVBs do not resemble early-stage autophagy organelles.

### 2.3.2. Colocalization with late-stage autophagy markers

Late-stage autophagy was probed with markers LAMP1 (lysosome-associated membrane protein-1) and CTSD (cathepsin D). LAMP1 is a membrane glycoprotein associated with late endosome, amphisome, and lysosome organelles, and thus can distinguish the later stages of the autophagic pathway from earlier stages (Chen *et al.*, 2008).
Double-label immunofluorescence microscopy revealed that LAMP1 immunoreactivity in AD hippocampal neurons undergoing GVD displayed a punctuate pattern throughout cytoplasm except in the immediate vicinity of GVBs, where it adopted an encircling colocalization pattern (Figure 2.4a-c). At the cellular level, 75 ± 7% of neurons undergoing GVD contained encircling LAMP1 immunoreactivity (Figure 2.3), which was significantly higher than the colocalization found for either early-stage autophagy marker LC3 or p62 ($p < 0.001$; Table 2.3). At the subcellular level, colocalization with GVBs averaged 32 ± 7% (Figure 2.2), which again was significantly higher than the colocalization observed with LC3 or p62 ($p < 0.001$; Table 2.4). These data are consistent with LAMP1 being a membrane protein associated with the surrounding membrane of the GVB vacuole. These data also indicate that GVBs are more closely related to late-stage than to early-stage autophagic organelles.

To extend this finding, the distribution of CTSD was investigated. CTSD is a major intracellular aspartic protease expressed in nearly all neurons and glial cells (Amano et al., 1995). It reportedly associates with late-stage autophagic organelles and endosomes, but with highest levels in lysosomes. For example, lysosomal CTSD levels have been reported to be nearly an order of magnitude above levels in late endosomes (Runquist and Havel, 1991; Casciola-Rosen et al., 1992). Thus CTSD immunostaining was expected to be a sensitive probe for lysosomes and their fusion products. Double-label immunofluorescence microscopy revealed that CTSD immunoreactivity in AD hippocampal neurons undergoing GVD adopted a punctuate pattern throughout the cytoplasm with occasional granular colocalization with GVBs (Fig 2.4d-f). At the
cellular level, 44 ± 10% of neurons undergoing GVD contained CTSD immunoreactivity colocalizing with GVBs (Figure 2.3). Although this was significantly higher than the colocalization found for either early-stage autophagy marker LC3 or p62 ($p < 0.001$; Table 2.3), it was significantly lower than for marker LAMP1 ($p < 0.01$; Table 2.3). At the subcellular level, 13 ± 4% of GVBs colocalized with CTSD (Figure 2.3), which again was significantly greater than the colocalization observed with LC3 or p62 ($p < 0.01$; Table 2.4) but significantly lower than the value for LAMP1 ($p < 0.05$; Table 2.4). Together with LAMP1 immunoreactivity, these data are consistent with GVBs being more closely related to late-stage than early-stage autophagic organelles. Because GVD granules are enveloped by LAMP1-containing membranes but contain limited amounts of CTSD, GVBs likely represent a vesicle similar to the amphisome in the autophagic pathway and point toward limited autolysosome formation as the approximate point of stalling.

2.3.3. Colocalization with endocytic protein CHMP2B

Amphisomes represent an intersection between endocytosis and autophagy, the two major pathways to the lysosome. Thus, under some circumstances, accumulation of amphisomes may be accompanied by endocytosis markers. A component of the endocytosis pathway, CHMP2B, has been reported to colocalize with GVBs at the cellular level (Yamazaki et al., 2010). CHMP2B is a component of the endosomal sorting complex required for transport-III (ESCRT-III), which is involved in endocytic trafficking of proteins (Raiborg and Stenmark, 2009; Urwin et al., 2009). ESCRT-III
drives the formation and specifically the scission of intraluminal vesicles in MVBs, and under certain conditions remain associated with them (Wollert et al., 2009). Therefore, the distribution of CHMP2B in AD hippocampus was quantified. Double-label immunofluorescence microscopy revealed that CHMP2B immunoreactivity adopted a punctate pattern having strong granular colocalization with GVBs (Figure 2.4g-i). At the cellular level, 98 ± 1% of neurons undergoing GVD contained granular overlapping CHMP2B immunoreactivity, which was significantly greater than the degree of colocalization found for any other investigated autophagy marker ($p < 0.001$; Table 2.3). At the subcellular level, colocalization with GVBs averaged 82 ± 3% (Figure 2.3), which again was significantly higher than the colocalization observed with the autophagy markers investigated above (Table 2.4). These data confirm that GVBs represent an organelle population with characteristics similar to amphisomes, but enriched with granular content from the endosomal pathway.

2.4. Discussion

On the basis of their ultrastructural morphology, GVBs have been proposed to bear some relation to autophagic organelles (Okamoto et al., 1991). Here we found that GVBs do indeed harbor autophagic marker proteins, especially those associated with the later stages of organelle maturation. Their epitope signature is consistent with organelles formed after fusion of autophagosomes with multivesicular bodies/late endosomes but before autolysosome maturation is complete. These data point to GVBs accumulating at the nexus between autophagy and endocytosis, the two major trafficking pathways to the
lysosome in neurons. Thus, GVBs could potentially arise from increased or aberrant flux through one or both of these pathways combined with a failure of lysosome-mediated clearance.

Of these three general possibilities, upregulation of autophagic flux is especially attractive as a response to intracellular aggregation and accumulation of tau protein in AD. However, it is not clear this contributes to GVB formation. First, the key suppressor of autophagy induction, the mTOR protein kinase (Diaz-Troya et al., 2008), is more heavily autophosphorylated at S2481 in AD relative to normal brain (Li et al., 2005), consistent with its activation (Soliman et al., 2010). Under these conditions, initiation of phagophore formation is predicted to be suppressed rather than activated in AD relative to normal brain. Second, tau aggregates do appear in the same neurons undergoing GVD (Hirano et al., 1968), but rarely (Holzer et al., 2002; Kannanayakal et al., 2006), and not within the GVB itself (Okamoto et al., 1991). Moreover, phospho-tau epitopes have been discovered within GVBs (Dickson et al., 1987; Bondareff et al., 1991; Mena et al., 1992; Ikegami et al., 1996), but the failure to detect many nonphospho-tau epitopes (Lübke et al., 1993) raises the issue of non-specific binding. In fact, GVBs bind several anti-phospho-epitope antibodies raised against diverse proteins while paradoxically failing to display the corresponding non-phospho epitopes (Kadokura et al., 2009). Third, p62, which could potentially facilitate autophagic clearance of ubiquitinated tau aggregates by tethering them to LC3 (Komatsu et al., 2007; Pankiv et al., 2007), only weakly colocalizes with GVBs despite its association with neurofibrillary tangles. Although a punctuate immunostaining pattern was reported for this protein in
AD brain (Kadokura et al., 2009), the results herein suggest that this pattern is unrelated to GVD. Finally, induction of autophagy in neurons is insufficient by itself to drive the accumulation of intermediate organelles (Boland et al., 2008). Together these observations suggest that GVBs form independently of neuritic lesions (Tomlinson and Kitchener, 1972) and are unconstrained by limitations in autophagic flux.

Aberrant flux through the endocytic system also is attractive as a contributor to GVD, since this system is at least partially responsible for the increased processing of APP to β-amyloid peptide observed in AD (Nixon, 2005). As reported previously (Yamazaki et al., 2010) and confirmed here, the endocytic system does appear to make a substantial contribution to the contents of GVBs, as indicated by strong colocalization of CHMP2B with the GVB granule. CHMP2B is a member of the ESCRT-III complex (Figure 2.1), which mediates membrane abscission during formation of the intraluminal vesicles within the multivesicular body (Wollert et al., 2009; Wollert and Hurley, 2010). It functions to recruit VPS4, an AAA+ ATPase required for the disassembly and recycling of ESCRT subunits (Lata et al., 2008). When this function is diminished through truncation mutagenesis, CHMP2B accumulates within MVBs instead of recycling (Wollert et al., 2009). Because VPS4 activity is subject to regulation under normal conditions, CHMP2B mislocalization and accumulation in disease may be secondary to aberrant VPS4 regulation rather than the rare genetic mutations that alter its primary structure and cause frontotemporal lobar degeneration (Urwin et al., 2009).

Finally, studies on cultured neurons reveal that inhibition of lysosome-mediated clearance is essential for accumulation of autophagic intermediates (Boland et al., 2008).
The defect that leads to GVB formation is not established, but our finding that GVBs colocalize more frequently with LAMP1 than lysosome marker CTSD suggest that diminished lysosome fusion efficiency is a candidate defect. This hypothesis is strengthened by cellular data in which fusion with the lysosome is inhibited by Rab7 knockdown. In these cells, multivesicular bodies become enlarged and more dense (Vanlandingham and Ceresa, 2009). Furthermore, cells that express a pathogenic CHMP2B mutation that results in frontotemporal dementia demonstrate specific disruption of the endosome-lysosome fusion by preventing recruitment of proteins necessary for fusion to occur, such as Rab7 (Urwin et al., 2010). Stalling of the autophagic pathway at an intermediate stage may have contributed to the LC3 staining pattern observed in this study, since prolonged exposure to cytosolic Atg4 would be expected to release LC3 from the outer membrane, resulting in a diffuse rather than membrane-associated appearance typically found in model systems (Kirisako et al., 2000).

The strong colocalization of Ckiδ with the GVB granule remains to be rationalized. Its presence suggests a selective enrichment relative to what might be expected from random bulk entrapment through lysosome delivery pathways. Casein kinase-1 family members, including Ckiδ, are established mediators of protein ubiquitination in cells through direct phosphorylation of E3 ligases or their recognition motifs (Winter et al., 2004; Galletti et al., 2009; Zhao et al., 2010). Monoubiquitination is a well characterized signal for targeting plasma membrane-associated proteins for endocytosis (Katzmann et al., 2002; Raiborg et al., 2003), and specific members of the casein kinase-1 family
regulate this reaction (Marchal et al., 2000). It is conceivable that phosphorylated and ubiquitinated cargoes, along with the catalyst Ckiδ, end up together in GVBs through the endocytic pathway. The nature of this relationship and the role of Ckiδ remains to be elucidated.

In summary, Ckiδ-immunoreactive GVBs most strongly colocalize with markers of the late autophagic and endocytic compartments relative to lysosomal protease CTSD or early-stage autophagy markers LC3 and p62. We speculate that GVBs arise from an increased or aberrant flux through the endocytic pathway that is inefficiently cleared owing to a defect in lysosome fusion efficiency (Figure 2.1). As a result, flux is routed into the autophagic pathway, which accumulates amphisome-like intermediates in abnormally large sizes owing to the same lysosome-fusion defect combined with mTOR-mediated suppression of phagophore formation. This hypothesis rationalizes the presence of both CHMP2B and ubiquitin in the GVB granules without a need for increased autophagic flux. The upstream events that lead to this phenotype may offer clues to regulatory events associated with sporadic AD, and whether GVD has toxic or protective effects on neurons.
2.5. Tables

Table 2.1. Case demographics
PMI, post mortem interval

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<th>Case (#)</th>
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<th>Gender</th>
<th>PMI (h)</th>
<th>Fixation</th>
<th>Braak Stage</th>
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<td>F</td>
<td>22</td>
<td>Formalin</td>
<td>IV</td>
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Table 2.2. Antibodies used in this study.

1Human Genome Organization nomenclature

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<tr>
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<th>Antibody</th>
<th>Source</th>
<th>Final Concentration</th>
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<td>128a</td>
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<td>2 (\mu)g/ml</td>
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Table 2.3. Pairwise comparison z-scores for cellular colocalization.
*,$ p < 0.05$, **,$ p < 0.01$, ***,$ p < 0.001$

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<td>4.03***</td>
<td>2.63**</td>
<td>-</td>
<td>5.66***</td>
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<td>63.4***</td>
<td>3.34***</td>
<td>5.66***</td>
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Table 2.4. Pairwise comparison z-scores for subcellular colocalization.
*,$ p < 0.05$, **,$ p < 0.01$, ***,$ p < 0.001$

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<th>CTSD</th>
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<td>-</td>
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<tr>
<td>CTSD</td>
<td>2.69**</td>
<td>2.88**</td>
<td>2.34*</td>
<td>-</td>
<td>13.4***</td>
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<td>23.6***</td>
<td>24.9***</td>
<td>6.27***</td>
<td>13.4***</td>
<td>-</td>
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2.6. Figures

Figure 2.1. Convergence of the autophagic and endocytic pathways destined for lysosome-mediated degradation.

In the autophagy pathway, p62-bound ubiquitinated organelles and cytosolic proteins targeted for degradation are engulfed by the phagophore, a double-layered membrane decorated by LC3-II, creating the autophagosome. In the endocytic pathway, internalized membrane proteins destined for lysosomal degradation are sorted into intraluminal vesicles of the MVB by sequential activity of ESCRTs 0, I, II, and III. CHMP2B is part of the ESCRT-III complex, which as a whole is specifically required for the abscission activity in the final stages of intraluminal vesicle formation. Mature MVBs fuse either with the autophagosome to form the amphisome or directly with the lysosome. Upon fusion with the MVB, the amphisome acquires vacuolar ATPases, which acidify the lumen, and lysosomal membrane proteins. Fusion of the amphisome with the lysosome, which donates degradative hydrolases, creates the autolysosome where complete degradation of the sequestered material occurs. See text for details.
Figure 2.2. Colocalization of GVBs with early stage autophagy markers.
Double-label confocal images (1x digital zoom, a-c, g-i; 4x digital zoom, d-f, j-l) of hippocampal sections stained with anti-Ckiδ antibody (green channel; a, d, g, j), which labels the GVB granule, and antibodies against LC3 (red channel; b, e) and p62 (red channel; h, k). Image overlays (c, f, i, l) highlight pixel overlap between Ckiδ and marker immunoreactivity in yellow. GVBs colocalized weakly with either LC3 or p62. In contrast, p62 colocalized with neurofibrillary tangles (h, arrow). Scale bars apply to each row.
Figure 2.3. Quantification of GVB colocalization.
Bars correspond to the overall mean percent colocalization of LC3, p62, LAMP1, CTSD, and CHMP2B immunoreactivity with GVBs ± SEM (n = 5 cases). Direct colocalization was defined as encircling and granular patterns. Statistical analysis of these data is summarized in Tables 2.3 and 2.4.
Figure 2.4. Colocalization of GVBs with late-stage autophagic and endocytic markers.

Double-label confocal images (4x digital zoom, a-f, j-l; 1x digital zoom, g-i) of hippocampal sections stained with anti-Ckiδ antibody (green channel) and antibodies against LAMP1, CTSD, and CHMP2B (red channel). High magnification panels j – l are three dimensional reconstructions, each composed of a stack of 25 optical slices acquired at 240 nm intervals. Image overlays highlight pixel overlap between Ckiδ and marker immunoreactivity in yellow. GVBs colocalized strongly with LAMP1, which stained the encircling GVB membrane (b, arrows), weakly with CTSD, which labeled a population of vesicles distinct from GVBs, and strongly with CHMP2B, which co-labeled the core granule. Scale bars apply to each row.
Chapter 3: Dual modification of Alzheimer’s disease PHF-tau protein by lysine methylation and ubiquitylation: a mass spectrometry approach

3.1. Introduction

Alzheimer’s disease (AD) is defined in part by the appearance of intracellular inclusions composed of the microtubule associated protein tau (Kuret, 2007). The mechanisms that drive tau lesion formation in the highly prevalent sporadic form of AD are not fully understood, but appear to involve abnormal post-translational modifications (PTMs) that influence tau function, stability, and aggregation propensity (Martin et al., 2011). For example, hyperphosphorylation of tau protein on certain hydroxy amino acids favors lesion formation by dissociating tau from its microtubule binding partner (Biernat et al., 1993; Bramblett et al., 1993) and by directly raising its rate and extent of aggregation (Alonso et al., 2001; Necula and Kuret, 2004b; Chang et al., 2011). Although tau phosphorylation state is mediated directly by phosphotransferases, it also is modulated by competing modifications on hydroxy amino acids such as O-linked β-N-acetylglucosaminylation (O-GlcNAcylation) (Liu et al., 2004a). The reciprocal relationship between these tau modifications is leveraged by O-GlcNAcase inhibitors, which by increasing O-GlcNAcylation, lower phosphorylation stoichiometry and depress neurofibrillary lesion formation (Yuzwa and Vocadlo, 2009). In addition to hydroxy
amino acids, Lys residues are modified on tau protein, and these too can influence tau metabolism and aggregation. For example, ubiquitylation of tau at Lys residues modulates intracellular tau levels (Petrucelli et al., 2004; Shimura et al., 2004), the magnitude of which affects both nucleation and extension phases of the aggregation reaction (Congdon et al., 2008). Together these observations suggest that tau aggregation is under complex regulatory control that involves crosstalk among diverse and sometimes competing PTMs.

To gain insight into the tau PTM signature most closely associated with neurofibrillary lesion formation at single amino acid resolution, we have begun mapping modifications on authentic, paired helical filaments (PHFs) isolated from AD brain using mass spectrometry methods, with special emphasis on Lys modifications (Cripps et al., 2006). Preliminary analysis identified K254, K311, and K353 within the tau microtubule binding repeat region as ubiquitylation sites that were at least partially occupied in PHFs (Cripps et al., 2006). Recently, acetylation was discovered as an other Lys-directed tau modification associated with tau-bearing lesions in AD and frontotemporal dementia (Min et al., 2010; Cohen et al., 2011). Tau acetylated in vitro resulted in occupation of diverse sites that overlapped with those we had determined to be ubiquitylated in disease (Min et al., 2010). Therefore acetylation is another candidate modification for regulating tau turnover indirectly through the ubiquitin-proteasome system (Min et al., 2010). Moreover, in other biochemical pathways, such as histone-mediated control of gene expression, certain acetylated Lys residues can alternatively be methylated, contributing to complex cross-talk among Lys and hydroxyl amino acid modifications (Latham and
Dent, 2007). These observations suggest that the web of tau PTMs is potentially complex, with Lys-directed modifications playing key regulatory roles with respect to rates of tau turnover and aggregation.

Here we extend our characterization of PHF-tau using mass spectrometry methods by expanding search criteria to include both acetyl- and methyl-Lys modifications. Although we found no evidence for tau acetylation/trimethylation at the level of detection available in our datasets, the results show that Lys monomethylation is a widespread PHF-tau modification. These data reveal tau methylation as a new tau PTM accompanying PHF deposition in vivo.

3.2. Materials and Methods

3.2.1. Subjects and tissue preparation

This study used only archival, de-identified post mortem brain tissue samples from autopsies performed with informed consent of each patient or relative via procedures approved by the relevant institutional committees (University of Rochester, NY). Paraformaldehyde-fixed brain tissue was obtained from six elderly subjects with a clinical diagnosis of AD (mean age 80 ± 10 yrs (SD)) that was confirmed on neuropathological evaluation in which the Consortium to Establish a Registry for AD (CERAD) age-adjusted criteria were met (Mirra et al., 1991), and from four pathologically normal controls (mean age 62 ± 10 yrs (SD)) who died without a history of neurologic or psychiatric disorder. All AD cases satisfied criteria for Braak stages V
or VI, while none of the control cases met pathological criteria for AD (Braak and Braak, 1991).

3.2.2. Affinity purification of PHF-tau and enzymatic digestion

PHF-tau was isolated from pooled, late-stage (Braak stages V or VI) AD neocortical regions by immunoaffinity chromatography (MC1 monoclonal antibody) as described previously (Jicha et al., 1997). PHF-tau was digested in solution in the presence of 40% methanol with either trypsin (Promega) or Lys-C (Sigma) followed by phosphopeptide enrichment (Immobilized gallium, Thermo Fisher Scientific) as detailed earlier (Cripps et al., 2006).

3.2.3. Reductive methylation of recombinant human 4R tau

Reductive methylation was done as described previously with minor modifications for optimization (Geoghegan, 2001). Briefly, lyophilized recombinant full-length human 2N4R tau (100 µg) was re-suspended in 100 µl of 0.1M citrate buffer (pH 6) and methylated (room temperature for 2 h) in the presence of 0.1 M sodium cyanoborohydride and 20 mM formaldehyde.

3.2.4. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Mass spectrometric analysis of PHF-tau was performed using an LTQ ion trap mass spectrometer controlled by Xcalibur v. 1.4 software (Thermo Electron) coupled online to a nanoflow XTreme Simple LC system (CVC Micro-Tech) as previously described.
Briefly, peptides were either loaded onto a trap column (Agilent Zorbax C18 guard column, or Michrom Bioresources peptide cap trap) or loaded directly into the sample loop with 95% solvent A (2% acetonitrile, 0.1% formic acid) and 5% solvent B (95% acetonitrile, 0.1% formic acid). A 60 min linear gradient of 5-25% solvent B was used to elute the peptides from the reverse phase column (150 mm x 75 µm, 5 µm 300 Å C18; CVC Micro-Tech).

The mass spectrometer was equipped with a nanospray ionization source (Thermo Electron) using an uncoated 10 µm i.d. SilicaTip PicoTip™ nanospray emitter (New Objective). The spray voltage of the mass spectrometer was 2.0 kV and the heated capillary temperature was 200 °C. The top five ions in each MS1 scan were selected for MS/MS fragmentation. After ions were selected for MS/MS fragmentation twice within 30 sec, they were dynamically excluded for 30 sec. An MS3 scan was triggered if, among the three most abundant ions in the MS/MS scan, a neutral loss of 98, 49, or 32.7 Da (corresponding to a loss of H$_3$PO$_4$ from 1+, 2+, or 3+ precursor ions, respectively) was detected. Other mass spectrometric data generation parameters were as follows: collision energy 24% (35% for MS3 scans), MS scan range 400 – 1800 m/z, minimum MS signal intensity 500 counts, minimum MS/MS signal intensity 100 counts, and MS/MS activation time 120 ms (30 ms for MS3 scans).

3.2.5. Analysis of mass spectrometric data
Spectra were searched against a UniProtKB human protein database (version Oct 5, 2010; 20,259 reviewed sequences; 75,498 non-reviewed sequences) using Bioworks 3.3.1
SP1 with the SEQUEST algorithm. Search parameters included 1.5 amu peptide mass tolerance, 1.0 amu fragment tolerance, static Cys +57 (carbamidomethylation) modification and the following differential modifications: Met +16 (oxidation); Ser, Thr, Tyr +80 (phosphorylation); Ser, Thr -18 (dehydroalanine and 2-amino-dehydrobutyric acid, respectively); Lys, Asp, Glu +14 (monomethylation); Lys +28 (dimethylation); Lys +42 (trimethylation/acetylation); and Lys +114 (ubiquitylation). Fully enzymatic (trypsin or Lys-C) peptides with up to two missed cleavages and charge-state dependent cross correlation (XCorr) scores ≥ 1.5, 2.5, and 3.0 for 1+, 2+, and 3+ peptides, respectively, and ΔCn > 0.1 were considered as initial positive identifications. All MS/MS and MS3 spectra of identified post-translationally modified peptides from the initial screening were subjected to manual verification.

All raw data from this study are freely available to the research community on our laboratory’s website, www.proteomeumb.org, which also serves as a data-sharing portal. The original raw data are also available from http://proteomecommons.org with the following Hash IDs: Tau trypsin digestion dataset: (Y297YWVSnnKcaY3jSnQkuCWj7tA1mls59uBBjjBpyqF5hOQ4lSmAuWNhtdC1EQ Csj5A7 XFM0/3zj9YvVUIIIluuwcoAAAAAAAAABrA==); Tau Lys-C digestion dataset: (pNmgHCue Fwyw3vtAgIdKGatW5G8weU7ArA/Fg+OiChNsahHADGSEQp7iUAOzouc81DMfWp eq7li1pDcjOkrs 1h9BHAAAAAAAABpg==).
3.2.6. Antibodies

Anti-tau mouse monoclonal antibodies Tau5 (LoPresti et al., 1995) and AT8 (Goedert et al., 1995) were obtained from Dr. L. I. Binder (Northwestern University Medical School) and Endogen (Woburn, MA), respectively. Rabbit polyclonal anti-methyl Lys (meK) antibody was obtained from Enzo Life Sciences (ADI-KAP-TF121; Plymouth Meeting, PA). Cy3-conjugated goat anti-rabbit IgG and Alexa Fluor 488-conjugated goat anti-mouse secondary antibodies were from Jackson Immuno Research Laboratories, Inc (West Grove, PA) and Invitrogen (Carlsbad, CA), respectively. Horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody used for Western Blot was from Kirkegaard and Perry Laboratories Inc (Gaithersburg, MD).

3.2.7. Immunohistochemistry

Coronal hippocampal tissue sections were cut (20 µm thickness) and processed for immunohistochemistry as described previously (Kannanayakal et al., 2006; Funk et al., 2011). Sections were rehydrated in PBST (2.7 mM KCl, 0.14 M NaCl, 8.1 mM Na₂HPO₄, 0.1% Tween-20, pH 7.4) and fixed (10 min) in ice cold methanol. After 3 x 5 min rinses in PBST, sections were blocked (1 hr at 19°C) with 5% goat serum diluted in PBST, then incubated (overnight at 4°C) with primary antibodies diluted in 2.5% goat serum (Tau5, 1 µg/ml; Anti-meK, 0.3 µg/ml; AT8, 0.1 µg/ml). After washing in PBST (3 x 10 min), sections were incubated (1 hr at 19°C) with fluorescent dye-labeled secondary antibodies (1.5 µg/ml Cy3-conjugated goat anti-rabbit IgG; 2 µg/ml Alexa Fluor 488-conjugated goat anti-mouse IgG). After washing in PBST (3 x 5 min), tissue was treated
(10 min at 19°C) with 0.1% Sudan Black B (EM Diagnostics, Gibbstown, NJ) in 80% ethanol to suppress lipofuscin autofluorescence (Schnell et al., 1999). Sections were then washed (2 x 5 min) in PBST and once for 5 min in 33 mM NaH$_2$PO$_4$, 162 mM Na$_2$HPO$_4$, pH 7.4. Coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and sealed with clear nail enamel. Labeled sections were viewed in a Leica TCS SL laser-scanning confocal system (40x oil HCX Plan Apo CS 0.75-1.25 NA or 100x oil HCX Plan Apo CS 0.70-1.40 NA objective lens) operated at wavelengths optimized for simultaneous detection of Alexa Fluor 488 ($\lambda_{\text{ex}} = 488$ nm; $\lambda_{\text{em}} = 500$-$530$ nm), and Cy3 ($\lambda_{\text{ex}} = 543$ nm; $\lambda_{\text{em}} = 560$-$600$ nm). Long wavelength fluorescence ($\lambda_{\text{ex}} = 633$ nm; $\lambda_{\text{em}} = 650$-$740$ nm) also was monitored to assess autofluorescence intensity. Digital confocal images were captured at 1x and 3x digital zoom, and stored in Tagged Image Format. Both secondary antibodies displayed minimal non-specific staining under these conditions as determined by immunostaining in the absence of primary antibodies. For immunoadsorption assay, primary antibodies anti-meK (0.3 µg/ml) and AT8 (0.1 µg/ml) were diluted in 2.5% goat serum containing 2000-fold molar excess of recombinant 2N4R tau (0.52 mg/ml) and incubated overnight (4°C) with agitation. Pellets were separated by centrifugation at 30,000 x g for 30 min, and the supernatant was used for immunohistochemistry as described above.

3.2.8. Analytical methods

The proportion of meK-positive lesions was estimated using the Wilson score method (Newcombe, 1998; Funk et al., 2011). Sufficient technical replicates (defined as Tau-
positive bodies at least 7 μm in both length and width) were counted from at least 5 fields of each case so that the Wilson 95% confidence interval for colocalization was <15%. Overall mean colocalization was then calculated as the average of all six biological replicate means and reported ± standard deviation.

3.3. Results

3.3.1. PHF-tau is methylated in its N-terminal projection and microtubule binding domains

To identify sites of Lys modification in PHF-tau, previously collected MS datasets obtained from two independent preparations of authentic AD-brain derived PHFs (digested with either trypsin or Lys-C proteases; [Cripps et al., 2006]) were interrogated using the SEQUEST database search algorithm programmed to identify unmodified Lys residues along with sites of monomethylation (K+14), dimethylation (K+28), trimethylation (K+42), acetylation (also K+42), and ubiquitylation (K+114). Sequence coverage in these datasets included 25 out of the 44 Lys residues present in the longest form of human brain tau protein (2N4R tau, Fig. 3.1). Although these search conditions confirmed three sites of ubiquitylation in the microtubule binding repeat region (K254, K311, and K353; [Cripps et al., 2006]), no evidence for either K+28 or K+42 masses was found, indicating that dimethyl-, trimethyl-, and acetyl-Lys were not present in the coverage area at the level of detection available in our datasets. However, robust monomethylation was identified at seven sites distributed throughout the tau sequence (Table 3.1). Three of the sites (K163, K174, and K180) reside within the proline-rich
region of the tau N-terminal projection domain, which mediates interactions with microtubule-associated proteins such as actin (He et al., 2009) and the Src homology 3 domain of plasma membrane-associated proteins including Src family kinases (Lee et al., 1998) and phospholipase Cγ (Reynolds et al., 2008). In contrast, K254, K267, and K290 are part of the first and second repeats of the microtubule-binding domain. They reside within or adjacent to the core β-sheet region of filamentous tau in vivo (Novak et al., 1993) and flank the “PHF6*” sequence that modulates fibrillation rate of recombinant monomeric 4R tau in vitro (von Bergen et al., 2000; Iliev et al., 2006; Li and Lee, 2006). Together these data reveal that PHF-tau is monomethylated in vivo, and that the major occupied sites distribute across protein segments known to mediate tau-protein interactions (including tubulin binding and self association).

To assess the relative abundances of methylated, ubiquitylated, and unmodified PHF-tau peptides, data were subjected to spectral counting, which measures the number of times a peptide is identified by MS/MS. Because spectral counts correlate linearly with protein abundance (Liu et al., 2004b), they have been employed for relative quantification in many label-free proteomic studies (Old et al., 2005; Zhang et al., 2006; Schmidt et al., 2007; Choi et al., 2008; Sardiu et al., 2008). Relative abundance was calculated by dividing the spectral count of a modified peptide by the sum of the spectral counts of all its forms (i.e., modified and unmodified). Results showed that the relative abundance of monomethylation varied among sites, from 12% (K290) to as high as 67% (K180 and K267) (Fig. 3.2). The relative abundance of ubiquitylation also varied, from
1% (K254) to 33% (K311) (Fig. 3.2). These data indicate that Lys modification occupancies are substantial in PHF tau.

3.3.2. Lys methylation participates in competitive crosstalk with ubiquitylation

The sites of monomethylation identified above directly overlapped with sites previously identified as being acetylated in vitro (K174, K180; (Min et al., 2010)) or ubiquitylated in vivo (K254; (Cripps et al., 2006)), raising the issue of competitive crosstalk at these residues. Although no Lys acetylation was detected at these sites in our datasets, it was possible to quantify relative methylation and ubiquitylation of K254. Methylated K254 was identified in Lys-C peptide aa241-254 from manually verified MS/MS spectra (Fig. 3.3). Relative to the 1+ charge state y-ions of unmodified aa241-254 peptide (Fig. 3.3a), the y-ions of methylated aa241-254 were shifted by +14Da (Fig. 3.3b), reflecting the mass of the methyl group (CH$_3$) added to (and the H atom lost from) the ε-amino group on the K254 side chain. The appearance of the +14 Da shift was not likely an artifact of Fischer esterification (i.e., chemical methylation of free carboxylic acid groups in Asp and Glu; (Jung et al., 2008; Lu et al., 2009)), because all MS/MS spectra were unambiguously assigned by SEQUEST (examples are shown in Supplemental Table 1). Moreover, care was taken to limit Fischer esterification conditions during sample preparation by maintaining the pH of digestion buffers >8, by avoiding exposure of samples to high concentrations of methanol in the presence of acetic acid, and by avoiding methanol as a co-solvent during LC separation of the peptides.
Ubiquitylated-K254 was identified in tryptic peptide aa243-257, which was resistant to protease cleavage at K254 owing to the isopeptide bond formed between ubiquitin and K254 (Fig. 3.3c). When aa241-254 and aa243-257 peptides were subjected to spectral counting, 41% of aa241-254 was monomethylated, whereas only 1% of aa243-257 was ubiquitylated (Table 3.2; Fig. 3.2). These data suggest that monomethylation competes directly with ubiquitylation at K254, and that monomethylation of this site is far more abundant than ubiquitylation in PHF-tau (Fig. 3.2, inset).

3.3.3. Lys methylation and potential cross talk with phosphorylation

Although several of the monomethylation sites on PHF-tau lie within five amino acid residues of phosphorylation sites identified in the literature (Hanger et al., 2009), only one peptide (aa258-267) was detected in our data sets that contained both a methylated Lys and a phosphorylated hydroxy amino acid. The phosphorylation site in this peptide, S262, functions as a regulatory gatekeeper for microtubule association, with site occupancy correlating with decreased binding affinity (Leger et al., 1997; Schneider et al., 1999). Its proximity to K267 allowed us to detect relative site occupancy within a short peptide sequence. Unmodified aa258-267 was identified as a Lys-C derived peptide from its MS/MS spectrum (Fig. 3.4a). Relative to the 1+ charge state γ-ions of this peptide, the γ-ions of methylated aa258-267 peptide were shifted by +14Da, reflecting the mass of the methyl group added to the ε-amino group of K267 (Fig. 3.4b). In contrast, the assigned γ7- and γ8-ions of aa258-267 phosphorylated at S262 were shifted by -18 Da, consistent with the dominant ion having undergone neutral loss of
$\text{H}_3\text{PO}_4$ by $\beta$-elimination ((Beausoleil et al., 2004); Fig. 3.4c). Peptide aa258-267 also was detected in a doubly modified (phosphorylated and methylated) form. Consistent with the behavior of phosphopeptides, the MS/MS spectrum of this peptide was dominated by a precursor ion that had undergone $\beta$-elimination (Fig. 3.4d).

To quantify the modification signature of these peptides, their relative abundance was measured by spectral counting (Table 3.3). Results revealed that the singly modified methyl-K267 peptide was the most abundant form (spectral count = 6; 35% of total aa258-267 peptide spectral count), and relative abundance followed the pattern: methyl-K267 ~ methyl-K267/phospho-S262 > phospho-S262 ~ un-modified K267/S262. Thus monomethylation at K267 was a more abundant PHF-tau modification than was phosphorylation at S262, with the latter being found more frequently in the presence of K267 methylation than found alone.

3.3.4. Methyl-Lys immunoreactivity associates with the neurofibrillary lesions of AD

In AD, PHF-tau accumulates within neurofibrillary lesions associated with neuronal cell bodies (NFTs), neuronal processes (neuropil threads), and the dystrophic neurites of neuritic plaques (Buee et al., 2000). To determine whether tau methylation correlated spatially with neurofibrillary pathology, sections of AD brain hippocampus were probed with a polyclonal antibody that binds meK-containing proteins along with well-characterized monoclonal anti-tau antibodies AT8 and Tau5 (LoPresti et al., 1995; Braak et al., 2006) in double label format. These tau antibodies were chosen for analysis because their epitopes are present during all phases of NFT development, including the
early pre- and intracellular-NFT stages (Shimazaki et al., 2005). The specificity of the anti-meK reagent was first tested on recombinant 2N4R tau preparations that were subjected to reductive methylation in vitro (see materials and methods). When subjected to SDS-PAGE, both non-methylated and methylated 2N4R tau migrated as single species, with the latter undergoing a band shift to 73 kDa (Fig. 3.5a). Rabbit polyclonal anti-meK antibody strongly labeled this species but not nonmethylated 2N4R tau (Fig. 3.5b). Binding specificity was further characterized by preadsorption assay, where antibodies were preincubated with 2000-fold molar excess of either non-methylated or methylated 2N4R tau before being used for immunohistochemical labeling of the hippocampal brain sections. When AT8 was preadsorbed under these conditions, neither non-methylated nor methylated tau preparations diminished its reactivity toward neurofibrillary lesions (detected by confocal immunofluorescence microscopy; Fig. 3.5c, f). These data were consistent with the established anti-phosphoepitope binding specificity of AT8 (Goedert et al., 1995). Anti-meK antibody also labeled neurofibrillary lesions in these sections, and like AT8, its labeling intensity was not affected by preadsorption with non-methylated 2N4R tau (Fig. 3.5d). However, labeling intensity was greatly diminished by pre-adsorption with methylated 2N4R tau (Fig. 3.5g). In contrast, analysis of pathologically normal brain sections revealed neither AT8-positive nor anti-meK-positive neurofibrillary lesions within similar hippocampal areas (Fig. 3.5i - k). Together these data are consistent with the anti-meK antibody being selective for Lys methylated proteins and specific to neurofibrillary lesions within the hippocampus.
Double-label confocal immunofluorescence studies were then extended to hippocampal brain sections prepared from six late-stage AD cases (case demographics are summarized in **Table 3.4**). Both AT8 (**Fig. 3.6a, d**) and Tau 5 (**Fig. 3.6g, j**) strongly labeled neurofibrillary lesions in these sections, including NFTs, neuropil threads, and neuritic plaques. At high magnification, NFTs immunolabeled with Tau5 displayed a fibrillar pattern throughout the lesions (**Fig. 3.6j**), while those labeled with AT8 also displayed a pattern of intense immunoreactivity enriched on the outer rims of the lesions (**Fig. 3.6d**). Rim staining, which has been seen previously with AT8 (Ikonomovic *et al.*, 2006), was particularly conspicuous at low fluorescence gain. In contrast, anti-meK immunoreactivity appeared diffusely distributed throughout the sections (**Fig. 3.6b, e, h, k**), with the most intense labeling correlating with both AT8- and Tau5-stained lesions, and with the pattern of reactivity resembling that of Tau5 with fibrillar staining throughout the lesion (**Fig. 3.6c, f, i, l**). Although background fluorescence was generally too high to detect neuropil threads with the anti-meK antibody, colocalization with particularly large or intense dystrophic neurites was occasionally seen (**Fig. 3.6g - i**). NFT labeling was robust, however, and so colocalization of meK immunoreactivity with this lesion was quantified in hippocampus CA1 region. Results showed that the majority of NFTs labeled with anti-meK antibody in all six cases (**Table 3.4**). Overall, anti-meK colocalization with AT8-labeled NFTs averaged 78 ± 11% (SD, *n* = 6 cases) whereas colocalization with Tau5-labeled NFTs averaged 79 ± 16% (SD, *n* = 6 cases). Together these data show that the methylation of PHF-tau identified through mass spectrometry is
widespread in this affected brain region, with most NFTs harboring anti-meK immunoreactivity in late-stage AD.

3.4. Discussion

Although certain familial tauopathies result from missense mutations in the tau gene (MAPT), AD pathogenesis is not associated with changes in tau amino acid sequence. Rather, tau lesion formation in sporadic AD is accompanied by PTMs that contribute to pathogenesis by modulating tau function, stability, and aggregation propensity. Because neurofibrillary lesion density correlates with neurodegeneration (Gomez-Isla et al., 1996; Josephs et al., 2008) and cognitive decline (Ghoshal et al., 2002; Royall et al., 2002; Giannakopoulos et al., 2003), a high priority in the AD field is to identify the PTM signature that drives neurofibrillary lesion formation in sporadic disease. Here we found that PHF-tau isolated from neocortical areas is methylated at at least seven distinct sites, and that the modification is widespread among NFTs in the CA1 region of hippocampus in late stage AD. These findings suggest that tau methylation is a component of the PTM signature associated with PHF-tau.

The direct effects of Lys methylation on tau function are not known, but in other proteins this modification is reported to promote protein-protein interaction by increasing the interaction radius of Lys side chains (Kim et al., 2008b) and by increasing the entropic driving force for its burial away from solvent (Sledz et al., 2010). For these reasons, reductive methylation is used to improve the performance of protein crystallization in preparation for diffraction experiments (Kim et al., 2008b). The
.localization of methylated residues within the microtubule binding repeat region in proximity to the PHF6 and PHF6* nucleation centers makes Lys methylation a candidate modification for directly affecting normal tau interactions with binding partners such as microtubules as well as abnormal interactions such as aggregation and PHF formation.

In addition to direct effects, Lys methylation also may modulate protein function through cross-talk with other PTMs. In its simplest form, crosstalk involves direct competition for occupancy of any single amino acid residue side chain. Here we found that K254 from PHF-tau isolated from AD brains was either ubiquitylated or methylated, with the latter strongly predominating. These data indicate that K254 methylation must occur at the expense of ubiquitylation, and thus is a candidate modification for regulating protein degradation via the ubiquitin-proteasome system (Min et al., 2010).

A second, more complex paradigm of crosstalk involves interactions between separate amino acid residues, where modification of one residue alters the ability of a second residue to bind its modifying enzyme. For example, tau phosphorylated by the microtubule affinity-regulating kinase 2 (PAR1/MARK2) is not recognized by the CHIP/Hsp90 E3 ligase complex and thus fails to be ubiquitylated or degraded (Dickey et al., 2008). Conversely, tau requires phosphorylation by GSK3β for efficient recognition and ubiquitylation by the CHIP/Hsp90 complex (Shimura et al., 2004). Here we found that K267 is methylated in PHF-tau, and that phosphorylation of S262 appears more frequently on peptides containing methylated rather than non-methylated K267. This observation raises the possibility of crosstalk between S262 phosphorylation, which strongly reduces the binding of tau to microtubules (Biernat et al., 1993; Schneider et al.,
1999), and K267 methylation. The impaired microtubule binding phenotype associated with S262 phosphorylation may be important for AD pathogenesis since this site is occupied early in NFT formation (Augustinack et al., 2002). Other sites of phosphorylation identified previously in PHF-tau (Hanger et al., 2009) (but not found in our datasets) also lie within close proximity to methylation sites. These include T175, T181, S184, S185, S258, and S289. Moreover, one projection domain methylation site, K44, is adjacent to a predicted calpain-catalyzed cleavage site in tau (Park and Ferreira, 2005). These data suggest that tau methylation is positioned to potentially engage in cross talk with multiple post translational modifications. It will be important to establish the temporal relationship among these modifications and the aggregation of tau during neurofibrillary lesion formation in AD. Mass spectrometry can complement high throughput immunohistochemical detection methods, which are sensitive to modification context (Fuchs et al., 2011), in this effort.

3.4.1. Comparison with previous studies

Previous characterization of PHFs prepared by differential centrifugation using amino acid analysis reported a low unmodified Lys content relative to predicted tau composition, consistent with extensive Lys modification, but failed to detect meK (Liu et al., 1991). The source of this discrepancy is not clear, but may relate to the sensitivity of amino acid analysis relative to MS and to the purity of the PHF preparations used for study.
Two recent studies reported that tau can be acetylated on Lys \textit{in vitro}, resulting in increased aggregation propensity and inhibition of tau degradation (Min \textit{et al.}, 2010; Cohen \textit{et al.}, 2011). \textit{In vivo} acetylation of K163, K174, K180 and K280 and accumulation within PHF-tau was claimed on the basis of antibody-based methods in tissue sections prepared from AD and frontotemporal lobar degeneration cases (Min \textit{et al.}, 2010; Cohen \textit{et al.}, 2011). On the basis of MS analysis, we did not detect tau acetylation even though residues K163, K174, K180 and K280 were resolved within our coverage area. These discrepancies could arise from any of several possibilities. One is that methylation occurs to a greater extent than acetylation. Consistent with this model, we found that K163, K174, and K180 were modified in PHF, but by monomethylation rather than acetylation. Combined with our detection of Lys ubiquitylation, these data suggest that the relative abundances of Lys methylation and ubiquitylation are likely higher than Lys acetylation in the PHF-tau preparation used herein. Another possibility is that PHF-tau contains substantial acetylation, but at sites distinct from those identified \textit{in vitro} or that reside within our current coverage area. Additional analysis of PHF will be required to test this hypothesis. A third possibility is that immunopurified PHF represents a subfraction of tau aggregates with greater SDS-solubility. For example, we find ((Cripps \textit{et al.}, 2006); \textbf{Fig. 3.1}) that immunopurified PHFs lack detectable phosphorylation at T175 and T181 that were reported qualitatively by others in some tissue/PHF preparations (Goedert \textit{et al.}, 1994; Hanger \textit{et al.}, 1998; Augustinack \textit{et al.}, 2002). In addition, Y394 phosphorylation has been reported in PHF-tau (Derkinderen \textit{et al.}, 2005; Tremblay \textit{et al.}, 2010), but this modification is present at rather low relative
abundance in the PHF-tau preparation used herein. It should be possible to resolve these possibilities in the future using mass spectrometry under conditions that provide quantitative information on modification occupancy.

In conclusion, we report biophysical evidence that seven Lys residues (K44, K163, K174, K180, K254, K267, and K290) in PHF-tau immunopurified from AD brain are monomethylated. The sites present opportunities for cross talk with other post-translational modifications, including direct competition with ubiquitylation and acetylation, and indirect interaction with phosphorylation and proteolysis. It will be important to determine the functional and temporal relationships among these established modifications in modulating the accumulation of PHF-tau in AD and other age-related neurodegenerative diseases.
3.5. Tables

Table 3.1 Lys methylation sites identified on PHF-tau.

Lys-C and trypsin in-solution digests of PHF-tau were analyzed by nanoflow LC-MS/MS and the data were searched against a human database using Bioworks with the SEQUEST algorithm. Amino acids in bold indicate identified methylated Lys residues. Amino acid (aa) residue numbering is based on the human 2N4R tau isoform (NCBI accession number NP_005901). Abbreviations for the PHF-tau domains are as follows: N = N-terminal projection domain; P = Pro-rich region; M = microtubule-binding domain; R1, R2 = repeat regions 1 and 2, respectively. Cross-correlation (XCorr) and Delta correlation (ΔCn) scores are two metrics in the SEQUEST algorithm used to assess the quality of candidate peptides assigned to MS/MS spectra. Peptides with charge-state dependent XCorr scores ≥ 1.5, 2.5, and 3.0 for 1+, 2+, and 3+ peptides, respectively, and ΔCn scores > 0.1 were the criteria for positive identifications.

<table>
<thead>
<tr>
<th>aa residues</th>
<th>Peptide</th>
<th>Methyl site</th>
<th>Charge state(s)</th>
<th>PHF-tau domain</th>
<th>XCorr score</th>
<th>ΔCn</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-44</td>
<td>DQGGYTMHQDQEGDTDAGLK</td>
<td>K44</td>
<td>3+</td>
<td>N</td>
<td>4.07</td>
<td>0.16</td>
</tr>
<tr>
<td>151-163</td>
<td>IATPRGAAPPQK</td>
<td>K163</td>
<td>2+</td>
<td>N, P</td>
<td>3.28</td>
<td>0.42</td>
</tr>
<tr>
<td>164-174</td>
<td>GQANATRIPAK</td>
<td>K174</td>
<td>2+</td>
<td>N, P</td>
<td>3.03</td>
<td>0.28</td>
</tr>
<tr>
<td>175-180</td>
<td>TPPAPK</td>
<td>K180</td>
<td>1+</td>
<td>N, P</td>
<td>1.59</td>
<td>0.33</td>
</tr>
<tr>
<td>241-254</td>
<td>SRLQTAPVMPDALK</td>
<td>K254</td>
<td>2+/3+</td>
<td>M, R1</td>
<td>3.71/3.55</td>
<td>0.41/0.24</td>
</tr>
<tr>
<td>258-267</td>
<td>SKIGSTENLK</td>
<td>K267</td>
<td>2+</td>
<td>M, R1</td>
<td>3.14</td>
<td>0.40</td>
</tr>
<tr>
<td>281-290</td>
<td>KLDLSNVQSK</td>
<td>K290</td>
<td>2+</td>
<td>M, R2</td>
<td>3.56</td>
<td>0.28</td>
</tr>
</tbody>
</table>
Table 3.2. Relative abundances of methylated and ubiquitylated PHF-tau peptides as assessed by spectral counts.
Spectral counts are defined as the number of times a peptide was identified by MS/MS. Amino acids in bold indicate identified methylated Lys residues. n.d. = not detected. % Modified is calculated based on the following spectral counts: modified peptide/(modified peptide + un-modified peptide). me, methylation; ub, ubiquitination.

<table>
<thead>
<tr>
<th>aa residues</th>
<th>Peptide</th>
<th>Modification site</th>
<th>Spectral count</th>
<th>% Modified</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Modified</td>
<td>Un-modified</td>
</tr>
<tr>
<td>25-44</td>
<td>DQGGYTMHQDQEGDTDAGLK</td>
<td>meK44</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>151-163</td>
<td>IATPRGAAPPGQK</td>
<td>meK163</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>164-174</td>
<td>GQANATRIPAK</td>
<td>meK174</td>
<td>2</td>
<td>n.d.</td>
</tr>
<tr>
<td>175-180</td>
<td>TPPAPK</td>
<td>meK180</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>241-254</td>
<td>SRLQTAPVPMPDLK</td>
<td>meK254</td>
<td>17</td>
<td>24</td>
</tr>
<tr>
<td>243-257</td>
<td>LQTAPVPMPDLKNVK</td>
<td></td>
<td>2</td>
<td>188</td>
</tr>
<tr>
<td>258-267</td>
<td>SKIGSTENLK</td>
<td>meK267</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>281-290</td>
<td>KLDLSNVQSX</td>
<td>meK290</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>299-317</td>
<td>HVPGGGSVQIVYKPVDLSK</td>
<td></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>350-369</td>
<td>VQSKIGSLDNITHVPGGGNK</td>
<td></td>
<td>2</td>
<td>49</td>
</tr>
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</table>
Table 3.3. Relative abundance of S262 phosphorylated and K267 methylated PHF-tau peptides.
Relative abundances were calculated by dividing the peptide spectral count for each peptide of interest by the total count for all peptides.

<table>
<thead>
<tr>
<th>Peptide (aa258-267)</th>
<th>Spectral count</th>
<th>% relative abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>unmodified</td>
<td>3</td>
<td>18%</td>
</tr>
<tr>
<td>phospho-S262</td>
<td>3</td>
<td>18%</td>
</tr>
<tr>
<td>phospho-S262, meK267</td>
<td>5</td>
<td>29%</td>
</tr>
<tr>
<td>meK267</td>
<td>6</td>
<td>35%</td>
</tr>
</tbody>
</table>
Table 3.4. Case demographics and marker colocalization in hippocampus CA1 region.
PMI, post mortem interval; n = number of lesions quantified

<table>
<thead>
<tr>
<th>Case (#)</th>
<th>Age (yrs)</th>
<th>Gender</th>
<th>PMI (hr)</th>
<th>Diagnosis</th>
<th>meK/AT8 colocalization ± SE</th>
<th>n</th>
<th>MeK/Tau5 colocalization ± SE</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>61</td>
<td>M</td>
<td>5.3</td>
<td>AD; Braak stage V-VI</td>
<td>86 ± 8%</td>
<td>43</td>
<td>92 ± 6%</td>
<td>36</td>
</tr>
<tr>
<td>2</td>
<td>81</td>
<td>F</td>
<td>--</td>
<td>AD; Braak stage V-VI, vascular involvement</td>
<td>89 ± 7%</td>
<td>39</td>
<td>89 ± 8%</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>81</td>
<td>M</td>
<td>4.1</td>
<td>AD; Braak stage VI, vascular involvement</td>
<td>78 ± 6%</td>
<td>154</td>
<td>82 ± 5%</td>
<td>168</td>
</tr>
<tr>
<td>4</td>
<td>82</td>
<td>F</td>
<td>4.0</td>
<td>AD; Braak stage V-VI</td>
<td>78 ± 7%</td>
<td>115</td>
<td>90 ± 6%</td>
<td>70</td>
</tr>
<tr>
<td>5</td>
<td>86</td>
<td>M</td>
<td>3.2</td>
<td>AD; Braak stage V-VI</td>
<td>59 ± 12%</td>
<td>70</td>
<td>52 ± 10%</td>
<td>97</td>
</tr>
<tr>
<td>6</td>
<td>87</td>
<td>M</td>
<td>2.5</td>
<td>AD; Braak stage V-VI</td>
<td>75 ± 14%</td>
<td>28</td>
<td>71 ± 13%</td>
<td>35</td>
</tr>
<tr>
<td>7</td>
<td>52</td>
<td>M</td>
<td>6.5</td>
<td>Control; myocardial infarct</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>8</td>
<td>57</td>
<td>M</td>
<td>7.5</td>
<td>Control; cardiac failure</td>
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<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>9</td>
<td>64</td>
<td>M</td>
<td>7.0</td>
<td>Control</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>10</td>
<td>75</td>
<td>M</td>
<td>8.2</td>
<td>Control; liver failure</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
3.6. Figures

Figure 3.1. Summary of modification sites identified by LC-MS/MS on immunopurified PHF-tau.

The sequence shown is that of human 2N4R tau (NCBI accession number NP_005901). Bold, sequence coverage; Red, PHF6 and PHF6* motifs; dashed lines, segments encoded by alternatively spliced exons 2, 3, and 10; underline, repeat region (as defined in (Goedert et al., 1989)); P, Phosphorylated sites; U, Ubiquitylated sites; me1, monomethylated sites identified from MS analysis reported herein and in (Cripps et al., 2006).
Figure 3.2 Relative abundance of PHF-tau methylation and ubiquitylation. Relative abundance was calculated based on the spectral counts of the modified peptide/(modified peptide + unmodified peptide). Only sites having total spectral counts > 3 are shown. Inset quantifies K254 methylation (41%) and ubiquitylation (1%). K254 was the most abundant methylated site identified on PHF-tau (spectral count = 17) and the only site on PHF-tau identified in methylated and ubiquitylated forms.
Figure 3.3. MS/MS characterization of K254 modification site.
Spectra for peptide aa241-254 derived from Lys-C cleavage of PHF-tau identified in (a) unmodified and (b) methylated (*, site of methylation) forms. (c) Spectrum for tryptic peptide aa243-257 identified in ubiquitylated form (^, site of ubiquitylation). Trypsin did not cleave ubiquitylated aa243-257 at K254 owing to its conjugation to the C-terminal di-Gly fragment of ubiquitin.
Figure 3.4. MS/MS identification of S262 phosphorylated and K267 methylated PHF-tau peptides.

Spectra for PHF-tau derived peptide aa258-267 identified in (a) unmodified, (b) monomethylated (*, site of methylation), (c) Ser phosphorylated (@, site of phosphorylation), and (d) doubly modified (*, site of methylation; @, site of phosphorylation) forms.
Figure 3.5. Anti-meK antibody specificity.
Aliquots of unmodified (-) and in vitro methylated (+) 2N4R tau were separated by SDS-PAGE (8% polyacrylamide) and either (a) stained with Coomassie Blue (500 ng tau proteins), or (b) subjected to immunoblot analysis with anti-meK antibody (100 ng tau protein). Molecular mass calibration markers (M) are shown in units of kDa. High-stoichiometry reductive methylation reduced recombinant 2N4R migration on SDS-PAGE. To further test specificity, antibodies AT8 (c, f; green channel) and anti-meK (d, g; red channel) were pre-adsorbed with either unmodified (c – e) or in vitro methylated (f – h) 2N4R tau, and then subjected to double-label confocal immunofluorescence microscopy on sections of AD hippocampus. Lesions labeled with both AT8 and anti-meK are marked by asterisks. AT8 labeling was unaffected by preadsorption with either unmodified or methylated tau, whereas anti-meK immunoreactivity was diminished by pre-adsorption with methylated (f – h; carets) but not unmodified tau (c - e). Neither AT8 (i) nor anti-meK (j) antibodies revealed neurofibrillary lesions in pathologically normal hippocampal sections.
Figure 3.6. Anti-meK immunoreactivity colocalizes with neurofibrillary lesions in AD hippocampus.

Double-label confocal images of hippocampal sections (CA1 region) stained with anti-tau mouse monoclonal antibodies AT8 (a, d; green channel) or Tau5 (g, j; green channel) along with the rabbit anti-meK antibody (b, e, h, k; red channel). Low magnification images (40x objective, 1x zoom) show colocalization of anti-meK immunoreactivity with NFTs (a, b; asterisks) and neuritic plaques (g, h; arrows). High magnification images (100x objective, 3x zoom) show morphology of typical NFTs (d, e and j, k). Image overlays highlight pixel overlap between anti-meK and AT8 (c, f) and Tau5 (i, l) immunoreactivity. Anti-meK immunoreactivity colocalized extensively with NFTs in all fields examined.
Chapter 4: Identification of Lysine Methylation as a Normal Post-Translational Modification of Tau and its Effect on Normal and Pathological Function

4.1. Introduction

Alzheimer’s disease (AD) is defined pathologically by the appearance of senile plaques, composed of amyloid beta peptide, and neurofibrillary tangles (NFTs), composed of the microtubule associated protein tau. Tau is highly expressed in neurons, its primary role to stabilize the neuronal cytoskeleton by interacting with microtubules. Microtubules, in turn, provide the tracks for motor proteins and thus, allow intracellular transport of vesicles, organelles, and protein complexes (Garcia and Cleveland, 2001). In solution, tau behaves as a random coil (Mandekow et al., 2007); however, in disease, the microtubule binding repeat (MTBR) regions undergo a conformational change to a β-sheet, acting as a seed for filamentous aggregation (von Bergen et al., 2000; von Bergen et al., 2001). The mechanisms responsible for tau aggregation are unclear, but appear to involve post translational modifications (PTMs) that alter the normal and pathological functions of tau.

In conjunction with alternative splicing of transcripts encoding the MTBR, tau-microtubule binding is highly regulated by phosphorylation state, which allows rapid modulation of microtubule dynamics. Axonal microtubules are not static, but their
relative stability is thought to be critical for proper function. However, axonal microtubules must remain dynamic in order to enable movement, axonal elongation, and microtubule orientation (Feinstein and Wilson, 2005). Because proper regulation of microtubule dynamics is essential for viability, cells exert powerful regulation over tau function via kinase and phosphatase activity on as many as 30 different residues (Buee et al., 2000). In disease, however, tau becomes hyperphosphorylated, modified by 3- to 4-fold more phosphate per mol protein (Ksiezak-Reding et al., 1992; Kopke et al., 1993). Hyperphosphorylation of tau depresses its affinity for tubulin, thereby reducing its ability to bind and stabilize microtubules, and thus increasing its free cytoplasmic concentration, a necessary condition in order to sustain filament formation (Lindwall and Cole, 1984; Biernat et al., 1993; Bramblett et al., 1993; Li et al., 2004). In disease, certain sites of hyperphosphorylation promote neurofibrillary lesion formation by supporting the conformational transition of free tau into an assembly-competent configuration, increasing filament nucleation, and decreasing filament dissociation (Chang et al., 2011).

Phosphorylation does not act in isolation, however; rather analyses have revealed a complex pattern of overlapping PTMs that are poised to act in concert. In order to determine the PTM signature most closely associated with NFT formation, we have begun mapping modifications on authentic paired helical filaments (PHFs) immunopurified from AD brain using mass spectrometry methods. We recently reported the discovery of Lys methylation as a previously unidentified PTM of PHF-tau (Thomas et al., 2012). Here we extend our characterization of tau methylation to soluble tau protein enriched from pathologically normal human brain. Analysis revealed that in
contrast to tau phosphorylation, which increases in disease, Lys residues are hypomethylated in the diseased state. Furthermore, using in vitro biochemical methods we have determined the effect of tau methylation on normal function of binding and stabilizing microtubules. We also establish the effect of methylation on tau aggregation propensity and kinetics.

4.2. Materials and Methods

4.2.1. Materials

Recombinant polyhistidine-tagged 2N4R tau was prepared as described previously (Carmel et al., 1996; Necula and Kuret, 2004c). [14C]Formaldehyde was obtained from Perkin Elmer (Waltham, MA, USA) with a specific activity of 54.8 Ci/mol. Purified tubulin was purchased from Cytoskeleton (Denver, CO, USA). Aggregation inducer Thiazine red (Chemical Abstract Service registry number 2150-33-6) was obtained from TCI America (Portland, OR, USA). Formvar/carbon-coated copper grids, glutaraldehyde, and uranyl acetate were obtained from Electron Microscopy Sciences (Fort Washington, PA, USA).

4.2.2. Preparation of tau from human control brain and HEK-293 cells

This study used only archival, de-identified post mortem brain tissue samples from autopsies performed with informed consent of each patient or relative via procedures approved by the relevant institutional committees (Ohio State University, Columbus, OH). Tau was enriched from brain tissue using methods detailed previously
Briefly, brain tissue obtained at autopsy lacking neuropathology was homogenized in 5 volumes of homogenization buffer (by weight) including phosphatase and deacetylase inhibitors (20 mM 2-(N-morpholino)ethanesulfonic acid (MES)/NaOH, pH 6.8, 80 mM NaCl, 1 mM MgCl₂, 2 mM EGTA, 0.1 mM EDTA, 1 mM PMSF, 10 mM Na₄O₇P₂, 20 mM NaF, 1 mM Na₃VO₄). The homogenate was separated by centrifugation (20 min at 27,000 x g). The supernatant was subjected to heat treatment (10 min boiling) in the presence of 0.5 M NaCl and 2% 2-mercaptoethanol. After heat treatment, the supernatant was collected (20 min at 27,000 x g) and treated with 2.5% (final concentration) of perchloric acid to remove acid-insoluble non-tau material. After centrifugation, the acid-soluble extract was treated with trichloroacetic acid (TCA) up to 20% (w/v) and the TCA-precipitated proteins were washed twice with cold acetone and allowed to dry. The TCA pellets were suspended in a buffer suitable for analysis.

Preparation of tetracycline-inducible 2N4R-expressing HEK-293 cells (TRex-293) was described previously (Bandyopadhyay et al., 2007). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 270 μM amphotericin B, and 170 μM streptomycin (37°C with 5% CO₂). Cells were grown under selection with 12 μM blasticidin, and treated with 1 mM tetracycline for 5 days to induce tau expression. For tau enrichment cells were rinsed once in cold phosphate-buffered saline (2.7 mM KCl, 0.14 M NaCl, 8.1 mM Na₂HPO₄, pH 7.4), collected with cell lifters, and centrifuged at 3000 x g for 5 min. Harvested cells
were then homogenized in 5 volumes of homogenization buffer (by weight) and processed as detailed above.

### 4.2.3. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Mass spectrometric analysis of tau enriched from cognitively normal brain was performed as previously described (Cripps et al., 2006; Thomas et al., 2012).

### 4.2.5. Reductive Methylation of recombinant human 2N4R tau

Reductive methylation was done as described previously with minor modifications (Jentoft and Dearborn, 1979; Geoghegan, 2001; Thomas et al., 2012). 2N4R tau was diluted to a final concentration of 20 µM in 0.1 M Na$_3$C$_6$H$_5$O$_7$, pH 6, 0.1 M NaBH$_3$CN, and 5 mM formaldehyde. For determination of methyl group incorporation, $[14C]$formaldehyde was diluted to a final specific activity of 0.88 Ci/mol. Reaction proceeded at 22°C for up to 60 minutes.

For analysis of radiolabel incorporation, products were separated from reactants by protein desalting spin columns (Pierce, Rockford, IL, USA) in 10 mM HEPES, pH 7.4, 50 mM NaCl. 20 µl of reaction product was added to 5 ml scintillation fluid (Cytoscint, Cole-Parmer, Vernon Hills, IL, USA) and radioactive counts measured. Reactions and measurements were done in triplicate and are reported as mean ± standard deviation (SD). Data was graphed in SigmaPlot and fit to an exponential rise to max equation:

$$y = a(1 - e^{-bx})$$

(Eq. 4.1)
where \( y \) is methyl group incorporation at time \( x \), and \( a \) represents the maximum methyl group incorporation (Bmax).

For mass spectrometry and biochemical analyses, reaction was prepared as described above with unlabeled formaldehyde. Methylated tau used for mass spectrometry samples were separated from reactants using protein desalting spin columns with 50 mM NaH\(_2\)HCO\(_4\), pH 7.8. Methylated tau used for biochemical analysis was prepared as described above, then stopped by addition of 50 mM glycine, and the products were then separated from reactants with disposable chromatography columns (BioRad, Hercules, CA, USA) in low salt assembly buffer (10 mM HEPES, pH 7.4, 50 mM NaCl). Fractions were collected as they eluted from the column and assayed for protein content by absorbance at 280 nm. When necessary, fractions were concentrated using Centricon Centrifugal Filter Units (Millipore, Billerica, MA, USA). Protein concentration was analyzed by bicinchoninic acid assay (Pierce, Rockford, IL, USA) in triplicate (not shown).

4.2.6. Microtubule binding assay

The microtubule binding assay was performed as described previously (Sun and Gamblin, 2009) in standard tubulin buffer (BRB80; containing 80 mM K-PIPES, pH 6.8, 1 mM MgCl\(_2\), 2 mM EGTA). Preformed microtubules were prepared in siliconized tubes by diluting tubulin dimer to 20 \( \mu \)M in BRB80, 1 mM GTP, and 1 mM DTT. 20 \( \mu \)M paclitaxel was added in 3 steps with 5 min incubations at 37°C. In siliconized tubes, either unmodified or reductively methylated tau protein at concentrations ranging from
0.25 to 8 μM was mixed with paclitaxel-stabilized microtubules at a final concentration of 2 μM tubulin dimer in 50 μl reaction volume. Samples were incubated at 22°C for 30 minutes, then layered over a cushion containing 40% glycerol and 20 μM paclitaxel in BRB80, and ultracentrifuged at 100,000 x g for 30 minutes to sediment microtubules and bound tau. The pellets were resuspended in 1X SDS sample buffer (50 mM Tris-Cl, pH 6.8, 100 mM dithiothreitol (DTT), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.1% bromophenol blue), and tubulin was separated from tau by SDS-PAGE. The amount of tau specifically bound to microtubules was determined by measuring optical density of the Coomassie blue-stained protein by ImageJ software (National Institutes of Health, Bethesda, MD, USA) and interpolating to the appropriate protein standard, then subtracting non-specific binding (pelleted tau when tubulin is excluded). Mols of bound tau were normalized to mols of polymerized tubulin dimer and free tau was determined by subtracting bound tau from total tau. Triplicate determinations were averaged, and bound tau / polymerized tubulin dimer was graphed versus free tau in SigmaPlot as mean ± SD. Data were fit to a four parameter logistic curve to determine the Bmax, AC50 and Hill slope.

\[
y = \min + \frac{\max - \min}{1 + (\frac{x}{AC50})^{\text{Hill slope}}}
\]

(Eq 4.2)

4.2.7. Tubulin polymerization assay

Tubulin polymerization assay was done as classically described (Mitchison and Kirschner, 1984; Bandyopadhyay et al., 2007). Either unmodified or reductively
methylated tau (2 µM) was incubated with 9 µM tubulin dimer in BRB80 with 1 mM DTT, 1 mM guanosine triphosphate, at 37°C. The time course of polymerization was followed by measuring absorbance at 340 nm every 1 min for 60 min using a Cary50 UV-Vis Spectrophotometer (Agilent, Santa Clara, CA, USA) with a Peltier single cell temperature control accessory. Change in absorbance at 340 nm was graphed as a function of time and fit to an exponential rise to max function in SigmaPlot (Equation 4.1) to calculate the extent \((a)\) and rate \((b)\) of polymerization. Assay and fittings were done in triplicate and parameters were averaged to yield a mean ± SD. Alternatively, Δ340 nm data points of each of three trials were averaged and plotted as a function of time ± SD.

4.2.8. Tau fibrillization assay

Tau filaments were formed from purified tau incubated without agitation in assembly buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, and 5 mM DTT) for up to 24 h (unless otherwise specified) at 37°C. Aggregation was initiated with Thiazine red (100 µM final concentration). Reactions were terminated with 2% glutaraldehyde, adsorbed to Formvar/carbon-coated copper grids, stained with 2% uranyl acetate, and viewed in a Tecnai G2 Spirit BioTWIN transmission electron microscope (FEI, Hillsboro, OR, USA) operated at 80 kV and 16,000-60,000X magnification. At least three viewing fields were captured for each reaction condition in which filaments >10 nm in length were counted and quantified with ImageJ. Total filament lengths of all resolved filaments per field are reported ± SD.
4.2.9. Critical concentration

Critical concentrations ($K_{\text{crit}}$) were determined by inverse prediction of the abscissa intercept ($\hat{x}$) of a plot of the concentration dependence of tau aggregation, as described previously (Congdon et al., 2008; Zhong et al., 2012) using the Taylor series expansion (Rice, 2007):

$$\hat{x} = \frac{\mu_y}{\mu_x} + \frac{1}{\mu_x^2} (\sigma^2_x \frac{\mu_y}{\mu_x} - \sigma_x \sigma_y r)$$

(Eq. 4.3)

Where $\mu_y \pm \sigma_y$ is the ordinate intercept ± SEE, $\mu_x \pm \sigma_x$ is the regression slope ± SEE, and $r$ is the regression correlation coefficient. The variance ($S^2$) was calculated as (Rice, 2007):

$$S^2 = \frac{1}{\mu_x^2} (\sigma^2_x \frac{\mu^2_y}{\mu_x} + \sigma^2_y - 2\sigma_x \sigma_y r \frac{\mu_y}{\mu_x})$$

(Eq. 4.4)

4.2.10. Dissociation kinetics

Assembled tau filaments prepared as described above were diluted 10-fold into assembly buffer containing 100 $\mu$M Thiazine red and incubated at 37°C. Aliquots were removed as a function of time up to 5 h post-dilution and assayed for total filament length. The disaggregation time course was fit to an exponential decay function to obtain $k_{\text{app}}$ as described previously (Kristofferson et al., 1980; Necula and Kuret, 2005; Zhong et al., 2012):

$$y = y_0 e^{-k_{\text{app}}t}$$

(Eq. 4.5)
where $y$ is the filament length at time $t$, $y_0$ is filament length at time zero, and $k_{\text{app}}$ is the pseudo-first order rate constant for the process.

The association rate constant $k_{e^+}$ was then determined from the relationship (Congdon et al., 2008):

$$K_{\text{crit}} = \frac{k_{e^-}}{k_{e^+}}$$

(Eq. 4.6)

assuming a two state model (i.e., all tau was either monomeric or incorporated into filaments).

4.2.11. Aggregation time series

Aggregation lag times, defined as the time when the tangent to the point of maximum aggregation rate intersects the abscissa of the sigmoidal curve (Evans et al., 1995), were obtained ± SE from each time series by Gompertz regression as described previously (Necula and Kuret, 2004c):

$$y = ae^{-e^-(t-t_i)/b}$$

(Eq. 4.7)

where $y$ is total filament length (50 nm cutoff) at time $t$, $t_i$ is the inflection point corresponding to the time of maximum growth rate, $a$ is the maximum total filament length, and $b = 1/k_{\text{app}}$, where $k_{\text{app}}$ is the proportional growth rate of the filament population in units of time$^{-1}$ (Winsor, 1932). Lag times, defined as the time when the tangent to the point of maximum polymerization rate intersects the abscissa of the sigmoidal curve (Evans et al., 1995), were calculated as $t_i - b$ (Winsor, 1932).
4.2.12. Statistical analysis

Estimated kinetic parameters were assumed to resemble normally distributed random variables ($X_i$) with mean $\mu_i$ and known standard deviation $\sigma_i$.

For pairwise comparisons, the probability ($p$) of differences among parameters with SD was assessed by one-way ANOVA and Bonferroni post hoc multiple comparison test using Prism 5 (GraphPad Software, Inc., La Jolla, CA). The probability ($p$) of differences between kinetic parameters with SEE was assessed by $z$-test:

$$z = \frac{x_1 - x_2}{\sqrt{(S_{x_1})^2 + (S_{x_2})^2}}$$  \hspace{1cm} (Eq. 4.8)

where $x_1 \pm S_{x_1}$ and $x_2 \pm S_{x_2}$ are the pair of estimates $\pm$ SE being compared, and $z$ is the $1-\alpha$ point of the standard normal distribution using JMP9.0 (SAS Institute, Cary, NC). The null hypothesis was rejected at $p<0.05$.

4.3. Results

4.3.1. Tau is highly methylated in microtubule binding region of pathologically normal brain.

Previous analysis identified seven sites of Lys-monomethylation spanning tau protein that had been immunopurified from PHFs isolated from AD brain (shown above amino acid sequence; Figure 4.1). To identify sites of Lys modification in normal biology, tau was enriched from cognitively normal human brain. Because unlike most
proteins, tau is heat-stable and acid-soluble, tau was enriched for by subtracting other proteins, and then concentrated by TCA precipitation. Resuspended tau was digested in-gel with trypsin and analyzed by nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS). Resulting data were searched against a human database using Bioworks with the SEQUEST algorithm programmed to identify unmodified Lys residues along with sites of monomethylation (K+14), dimethylation (K+28), and trimethylation (K+42) as well as acetylation (also K+42). These search criteria identified eleven unique sites of modification with evidence of mono- and di-methylation (Table 4.1). Two sites are found in the N-terminal projection domain, while nine are found within the MTBR (shown below amino acid sequence; Figure 4.1). Notably, K311, found in the dimethylated form, resides within the “PHF6” sequence, which has been shown to modulate the fibrillization rate of recombinant monomeric tau in vitro (von Bergen et al., 2000; Iliev et al., 2006; Li and Lee, 2006). The presence of tau methylation in normal biology was further examined by enriching for tau from HEK293 cells that express human 2N4R tau under a tetracycline-inducible promoter (Bandyopadhyay et al., 2007). In this system, three sites of dimethylation were identified, two of which lie within the MTBR and overlap with sites found to be methylated in pathologically normal human brain (HEK; Figure 4.1). Together, these data reveal that tau is a highly methylated protein in normal biology and decreased in the AD-state.

4.3.2. Preparation of reductively methylated tau.
In order to determine the effect of varying degrees of Lys methylation on the normal and pathological functions of tau, methylated tau was prepared *in vitro* by reductive methylation. Reductive methylation is a well-characterized method for Lys-specific protein methylation (Means and Feeney, 1968; Borch *et al*., 1971). Rate and extent of tau methylation was monitored by incorporation of $[^{14}\text{C}]$-labeled methyl groups, which is donated by $[^{14}\text{C}]$-formaldehyde during the reductive methylation reaction. Stoichiometry of methyl group incorporation was calculated by measuring the level of $^{14}\text{C}$ incorporated into the known amount of tau. Under the conditions indicated, methylation proceeded in a hyperbolic fashion, rising to a maximum of approximately 25 mol methyl per mol tau. At 7, 15, 30 and 60 minutes, approximately 5, 10, 16, and 22 mol methyl were incorporated per mol tau, respectively, as calculated by fitting the progress curve to an exponential rise to maximum (*Equation 4.1; Figure 4.2*). The sites of methyl group incorporation at high methylation stoichiometry (22 mol methyl per mol tau) were determined by LC-MS/MS analysis (*ReMe, Figure 4.1*). Search criteria identified nine sites of modification, each of which was identified in either the mono- or di-methylated form, thus accounting for eighteen of the twenty-two mol methyl determined by $^{14}\text{C}$ incorporation. Furthermore, five of the nine sites modified by reductive methylation are also modified in normal biology, four of which lie within the MTBR. Importantly, all reductively methylated sites within the MTBR are also modified in cognitively normal biological samples, thus facilitating biochemical analysis of normal and pathological tau function, both of which are mediated by the MTBR.
4.3.3. Physiological levels of tau methylation do not inhibit normal functions of tau.

One of the normal functions of tau is the binding and stabilization of microtubules (Weingarten et al., 1975; Witman et al., 1976; Drubin and Kirschner, 1986; Esmaili-Azad et al., 1994). Certain PTMs, including phosphorylation, can decrease the affinity of tau for microtubules and its ability to induce the polymerization of tubulin dimers into microtubules (Biernat et al., 1993; Yoshida and Ihara, 1993; Lu et al., 1999; Cho and Johnson, 2003). To determine the effect of methylation on tau function in normal biology, we examined tubulin binding and polymerization using in vitro methods. Tubulin binding was assayed using preformed paclitaxel-stabilized microtubules incubated with either unmodified or reductively methylated tau, then ultracentrifuged, the supernatant removed, and the pellet resuspended in SDS reducing buffer. Protein was separated by SDS-PAGE and the amount of protein estimated by Coomassie blue staining (a representative gel depicting unmodified tau binding is shown in Figure 4.3A). Non-specific binding was estimated by withholding tubulin from the protocol and was subtracted from the total bound tau, yielding specifically bound tau, which was normalized to the measured polymerized tubulin. Experiments were done in triplicate and the average of specifically bound tau / polymerized tubulin was plotted ± standard deviation versus free tau ± standard deviation (Figure 4.3B). Data were fit to a four parameter logistic curve with SigmaPlot (Equation 4.2) to estimate the binding maximum (Bmax; Figure 4.3C), binding affinity (AC50; Figure 4.3D), and Hill slope (Figure 4.3E). Calculated parameters are listed in Table 4.2. Overall, Bmax was unchanged, even with high methylation stoichiometry, and although AC50 increased with
methylation, this change was not significant at \( p > 0.05 \) with either 5 or 22 mol/mol incorporation. However, Hill slope decreased two-fold with 5 mol/mol methylation, suggesting decreased tight binding affinity (Figure 4.3E). Hill slope was not further depressed with 22 mol/mol methylation, however, suggesting that the site responsible for disrupting microtubule binding is modified early in the reaction process. This agrees with previous data regarding microtubule binding with tau phosphorylation. Tau modified with six or seven pseudophosphorylation sites did not exhibit significantly different levels of microtubule binding versus single or double mutants (Sun and Gamblin, 2009), thus suggesting that microtubule binding is regulated in a site-specific fashion, rather than levels of modification.

To determine whether the modest decrease in microtubule binding affinity is reflected with impaired polymerization, microtubule assembly was assayed \textit{in vitro} using classic methods. Either unmodified or reductively methylated tau was incubated at 37\(^\circ\)C with purified tubulin under conditions described in materials and methods, and the rate and extent of tubulin polymerization was measured by change in light absorbance at 340 nm. As shown in Figure 4, tubulin incubated at 37\(^\circ\)C in the absence of tau failed to appreciably polymerize. With the addition of unmodified tau, tubulin polymerized in an exponential fashion rising to a maximum of 1.14 absorbance units (AU) at a rate of 1.02E-3 s\(^{-1}\). While low levels of methylation (5-10 mol/mol) promoted tubulin polymerization identical to unmodified controls, high levels of methylation (16-22 mol/mol), which are likely super-physiological, depressed the ability of tau to promote the extent microtubule polymerization in a dose-dependent fashion (Figure 4.4), though
rate of microtubule polymerization remained unchanged (summarized in Table 4.3). Together, these data indicate that low levels of methylation do not inhibit the normal tau functions.

4.3.3. Lys methylation impedes self-aggregation of tau.

The self-aggregation of tau into paired helical filaments is a well-documented correlate of AD. In order to determine the effect of methylation on the pathological function of tau in self-aggregation, tau was incubated in the presence of Thiazine red aggregation inducer under near physiological conditions of pH, reducing conditions, and ionic strength. Under these conditions unmodified 2N4R tau forms twisted ribbons (Chirita et al., 2005) with a mass-per-unit length similar to authentic brain-derived PHFs (Congdon et al., 2008). Tau aggregation was monitored by capturing images using transmission electron microscopy (TEM). Low magnification images demonstrate that while unmodified tau readily aggregates (Figure 4.5A), methylation impairs aggregation propensity in a dose-dependent manner (Figure 4.5B-E). High magnification images depict similar morphology of both unmodified and methylated tau filaments (Figure 4.4A, B insets). These data indicate that methylated tau shares the fundamental aggregation characteristics of unmodified tau and can be studied at physiological bulk tau concentrations in the presence of Thiazone red inducer.

In order to quantify the effects of methylation on relative aggregation propensity, the critical concentration \( K_{\text{crit}} \) of unmodified or methylated tau was estimated in the presence of Thiazone red inducer. In nucleation-dependent reactions, \( K_{\text{crit}} \) approximates
the equilibrium dissociation constant for elongation, $K_e$ (Congdon et al., 2008). $K_{\text{crit}}$ also represents the highest protein monomer concentration that does not support aggregation and is thus, estimated from the abscissa intercept of the tau concentration dependence plateau fibrillization (Figure 4.6A). Results show that 5 mol/mol methyl incorporation increases the critical concentration nearly three-fold relative to unmodified tau (Table 4.4; Figure 4.6B). These data indicate that Lys methylation depresses aggregation propensity, in part, by increasing the minimum concentration of tau needed to support fibril formation.

$K_{\text{crit}}$ approximates the ratio of dissociation ($k_e$) and association ($k_{e+}$) rate constants for filament elongation (Equation 4.6). Thus, modulation of $K_{\text{crit}}$ may result from changes in either filament stability ($k_e$), efficiency of monomer association with filament ends ($k_{e+}$), or both. To differentiate between these possibilities, $k_e$ was estimated for both unmodified and 5 mol/mol methylated tau by diluting preassembled filaments below their $K_{\text{crit}}$ and estimating the initial rate of filament shortening by electron microscopy. Loss of filament followed first-order kinetics as predicted for endwise depolymerization from a Poisson-like length distribution (Necula and Kuret, 2005; Congdon et al., 2008) (Figure 4.7A). The dissociation elongation constant $k_e$ was derived from the disaggregation rate of both unmodified and methylated tau using the established relationship between tau mass and filament length (Congdon et al., 2008). Rate constant $k_{e+}$ was then calculated from estimates of $k_{e+}$ and $K_{\text{crit}}$ for each isoform using Equation 4.6. Parameter values are summarized in Table 4.4. Pairwise comparisons demonstrated that 5 mol/mol methylation increases $k_e$ nearly two-fold and decreases $k_{e+}$ about one and a half-fold.
relative to control (Figure 4.7B), suggesting that both filament elongation and stability are decreased by methylation.

In the presence of Thiazine red inducer, the aggregation reaction is driven by rapid equilibrium of assembly-competent monomers with a thermodynamic nucleus, defined as the least stable species reversibly interconverted with monomer (Ferrone, 1999). Since elongation can proceed efficiently only after the nucleus has formed, the rate of aggregation depends on nucleation rate as well as protein concentration and the rate of elongation ($k_e$ and $k_{e^+}$). In order to determine whether methylation affects nucleation rate, tau aggregation time course was quantified for both unmodified and 5 mol/mol methyl tau at constant supersaturation in the presence of Thiazine red. Under these conditions, differences in reaction rate primarily reflect differences in rates of nucleation and protein concentration (Fesce et al., 1992). Both reaction curves displayed lag, exponential growth, and equilibrium phases (Figure 4.8A). Data was fit to a three-parameter Gompertz growth function to calculate lag time (Equation 4.7; summarized in Table 4.4), which vary inversely with nucleation rate (Evans et al., 1995). Data reveal that unmodified tau aggregated with a slight but significantly shorter lag time relative to methylated tau, despite being present at lower bulk concentrations (Figure 4.8B). These data suggest that methylation modestly decelerates the nucleation phase of the tau aggregation reaction, though it has a greater effect on the rate of filament extension.
4.4. Discussion

Genetic mutations in the MAPT gene that encodes tau are responsible for certain rare forms of dementia collectively known as tauopathies; however, no such mutations have been identified responsible for AD. Rather neurofibrillary lesion formation is associated with changes in the tau PTM signature. The most well established tau PTM is phosphorylation, which increases 3- to 4-fold with disease onset; however phosphorylation does not exist in isolation. Rather, analyses have revealed a complex pattern of overlapping PTMs acting in a coordinated fashion to modulate tau activity. Recently we identified methylation as a novel tau PTM (Thomas et al., 2012), and here we show that in contrast to phosphorylation, methylation is decreased in the disease state. We propose that tau PTMs, including phosphorylation and methylation, act in concert to affect both normal and pathological functions of tau.

4.4.1. Implications for Microtubule Dynamics

Microtubule binding and stabilization remain the most extensively studied functions of tau in normal biology, though whether or not these are vital is still debated. Studies have revealed that the majority of tau in the cell is bound to microtubules, and, as shown here and elsewhere, this microtubule binding activity promotes their assembly and stability. However, in cell culture and in vivo, tau colocalizes with those microtubules that are most dynamic (Kempf et al., 1996; Fanara et al., 2010). Thus, microtubule stabilization, which can be compensated for by another microtubule-associated protein found in axons, MAP1B, may not be a critical function of tau in vivo. In fact, tau has
numerous binding partners, including signaling molecules (Reynolds et al., 2008), cytoskeletal elements (Fulga et al., 2007), and lipids (Hwang et al., 1996; Jenkins and Johnson, 1998; Reynolds et al., 2008), suggesting that it is a multifunctional protein.

A prominent theory suggests that disease may be caused by loss of tau function due to hyperphosphorylation and sequestration of soluble tau. However, considering that hyperphosphorylation occurs in fetal development (Seubert et al., 1995) and hibernation (Arendt et al., 2003) without filamentous aggregation, hyperphosphorylation of tau per se does not cause neurodegeneration. Furthermore, several mouse lines with MAPT knocked out have been developed, and for the most part, exhibit normal behavior throughout most of their lives (Ikegami et al., 2000; Roberson et al., 2007; Dawson et al., 2010; Ittner et al., 2010).

Here we show that methylation has a relatively modest effect on microtubule affinity: the Hill slope decreases by about 2-fold, but Bmax and AC50 do not significantly change (Figure 4.3). By comparison, some sites of pseudophosphorylation decrease binding affinity as much as three-fold (Sun and Gamblin, 2009) and certain tauopathic mutations decrease microtubule binding affinity by two-fold under similar experimental conditions (Hong et al., 1998). Provided that the dissociation equilibrium constant for binding remains lower than the concentration of available tau binding sites on the microtubules, small changes in affinity may not appreciably affect free tau concentrations. For example, wild type or tauopathic mutant tau microinjected at physiological concentrations into Michigan Cancer Foundation 7 (MCF7) cells colocalize with tubulin similarly (Bunker et al., 2006). Therefore, it is unlikely that any loss of tau
function caused by methylation would significantly impact neuronal health, and
differences in dynamics most likely reflect intrinsic mechanistic differences rather than
differential binding.

4.4.2. Implications for Aggregation Mechanism

Tau methylation may exert its benefit by potently inhibiting tau aggregation into
pathological filaments. Tau aggregation within cells is a complex process that is
modulated by both intrinsic aggregation propensity and modulation by other cellular
factors. We have proposed that the tau aggregation pathway involves four principal steps
that must be overcome in order for filamentous aggregates to accumulate in disease
(Congdon et al., 2008) (Figure 4.9). First, the concentration of cytoplasmic free tau must
rise to exceed the minimal tau concentration necessary to support aggregation. This can
be accomplished by increased MAPT expression, decreased tau degradation, or decreased
affinity of tau for microtubules. As discussed above, although there is slightly decreased
affinity for microtubules, it is still within the realm of tight binding, and likely does not
contribute to changes in free tau concentration. The potential role of tau methylation in
protein degradation is intriguing considering that methylation and ubiquitylation compete
for Lys residue site occupancy (Figure 4.1); however, the actual contribution of tau
methylation to protein degradation is currently unknown. It is interesting that two sites of
methylation in normal human brain (K254 and K311) are found to be ubiquitylated in AD
brain. The dynamic modulation of these sites and their relative function in health and
disease are still to be completed.
The second step of the tau aggregation pathway involves a conformational change to an assembly competent state (Figure 4.9). Because high concentrations (up to 100 μM) free tau alone are insufficient to support aggregation in vitro (Ko et al., 2002), this is proposed to be a barrier to aggregation. Phosphorylation of tau is thought to induce local polyproline II helix conformation (Bielska and Zondlo, 2006), and assist in overcoming the resistance of monomeric tau to aggregate. Although the conformational changes associated with methylation of tau are unknown, methylation of other proteins does not appear to significantly alter protein secondary structure (Rypniewski et al., 1993). However, increasing methyl stoichiometry, either by the number of sites or the nature of methylation (i.e., mono, di, or tri), increases a protein’s hydrophobicity (Taverna et al., 2007). It has been hypothesized that the unfolded nature of tau can be attributed mostly to the lack of hydrophobicity and that aggregation is largely determined by ionic interactions (Jeganathan et al., 2008). Therefore, it is possible that methylation inhibits tau aggregation by stabilizing it in a conformation resistant to aggregation.

Once aggregation-competent conformations are adopted, the rate-limiting step in filament formation becomes dimerization (Congdon et al., 2008), which is energetically disfavored at physiological tau concentrations, and therefore, a third point of control (Figure 4.9). Reductive methylation of tau decreases aggregation propensity consistent with a slight deceleration of filament nucleation rate. The final step in tau fibrillization is mediated by filament extension. Although not a rate-limiting step, equilibria at filament ends dictate the minimal concentration of tau required to support aggregation. Reductive methylation of tau significantly decreased filament elongation by decreasing the rate at
which monomers associate with filament ends as well as increasing the rate at which monomers dissociate with filament ends. Together, these results indicate that Lys methylation of tau acts in opposition to phosphorylation and certain tauopathic mutations, which promote tau filament formation.

4.5. Conclusions

Altogether these data suggest that tau is highly methylated in normal biology and could depress neurofibrillary lesion accrual though the inhibition of tau filament formation. This work establishes the role of methylation as a modulator of tau protein, and emphasizes the overlapping and sometimes contradicting PTM signature that act in concert to regulate both normal and pathological functions of tau. Furthermore, this suggests tau methylation may be a novel target for pharmacological efforts in disease-modifying treatments of tauopathies.
4.6. Tables

Table 4.1 Lys methylation sites identified on normal tau.
Trypsin in-gel digests of normal tau were analyzed by nanoflow LC-MS/MS and the data were searched against a human database using Bioworks with the SEQUEST algorithm. Bold = methylated Lys residues. Amino acid (aa) residues are based on 2N4R human. Abbreviations for tau domains: N = N-terminal projection domain; P = Pro-rich region; M = microtubule-binding domain; R1, R2, R3, R4 = repeat regions 1, 2, 3, and 4 respectively; C = C-terminal projection domain. Abbreviations for modifications are as follows: me1, monomethyl; me2, dimethyl

<table>
<thead>
<tr>
<th>aa residues</th>
<th>Peptide</th>
<th>Methyl site</th>
<th>Modification</th>
<th>Tau domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-44</td>
<td>KDQGGYTMHQDQEDTDAGLK</td>
<td>K24</td>
<td>me2</td>
<td>N</td>
</tr>
<tr>
<td>25-44</td>
<td>DQGGYTMHQDQEGTDAGLK</td>
<td>K44</td>
<td>me1</td>
<td>N</td>
</tr>
<tr>
<td>243-257</td>
<td>LQTAPVPMPDKNVK</td>
<td>K254</td>
<td>me2</td>
<td>M, R1</td>
</tr>
<tr>
<td>258-267</td>
<td>SKIGSTENLK</td>
<td>K259</td>
<td>me1</td>
<td>M, R1</td>
</tr>
<tr>
<td>281-290</td>
<td>KLDLSNVQSK</td>
<td>K281</td>
<td>me2</td>
<td>M, R2</td>
</tr>
<tr>
<td>281-290</td>
<td>KLDLSNVQSK</td>
<td>K290</td>
<td>me2</td>
<td>M, R2</td>
</tr>
<tr>
<td>306-317</td>
<td>VQIVYKPVDLSK</td>
<td>K311</td>
<td>me2</td>
<td>M, R3, PHF6</td>
</tr>
<tr>
<td>306-317</td>
<td>VQIVYKPVDLSK</td>
<td>K317</td>
<td>me2</td>
<td>M, R3</td>
</tr>
<tr>
<td>322-343</td>
<td>CGSLGNIHHKPGGGQVEVKSEK</td>
<td>K343</td>
<td>me2</td>
<td>M, R4</td>
</tr>
<tr>
<td>350-369</td>
<td>VQS\textbf{K}GSLDNITHVPGGGNK</td>
<td>K353</td>
<td>me2</td>
<td>M, R4</td>
</tr>
<tr>
<td>354-369</td>
<td>IGSLDNITHVPGGGNK</td>
<td>K369</td>
<td>me2</td>
<td>M, R4</td>
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</tbody>
</table>
Table 4.2. Lys methylation depresses microtubule binding affinity.

<table>
<thead>
<tr>
<th>Tau Species</th>
<th>Binding Max ± SEE (mol tau / mol tubulin dimer)</th>
<th>AC50 ± SEE (µM)</th>
<th>Hill slope ± SEE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified</td>
<td>1.72 ± 0.08</td>
<td>590 ± 63</td>
<td>2.32 ± 0.45</td>
</tr>
<tr>
<td>5 mol/mol</td>
<td>1.69 ± 0.14</td>
<td>956 ± 186</td>
<td>1.22 ± 0.19</td>
</tr>
<tr>
<td>22 mol/mol</td>
<td>1.49 ± 0.20</td>
<td>871 ± 294</td>
<td>1.15 ± 0.29</td>
</tr>
</tbody>
</table>

Table 4.3. Effect of Lys methylation on microtubule assembly
au, absorbance units

<table>
<thead>
<tr>
<th>Tau Species</th>
<th>Rate of Polymerization ± SD (sec⁻¹)</th>
<th>Extent of Polymerization ± SD (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Tau</td>
<td>1.07E-03 ± 8.39E-04</td>
<td>0.15 ± 0.05</td>
</tr>
<tr>
<td>Unmodified</td>
<td>1.02E-03 ± 7.36E-05</td>
<td>1.14 ± 0.08</td>
</tr>
<tr>
<td>5 mol/mol</td>
<td>8.34E-04 ± 2.62E-04</td>
<td>1.32 ± 0.19</td>
</tr>
<tr>
<td>10 mol/mol</td>
<td>9.24E-04 ± 3.07E-04</td>
<td>1.23 ± 0.11</td>
</tr>
<tr>
<td>16 mol/mol</td>
<td>1.28E-03 ± 4.81E-04</td>
<td>0.70 ± 0.05</td>
</tr>
<tr>
<td>22 mol/mol</td>
<td>1.14E-03 ± 1.12E-03</td>
<td>0.35 ± 0.13</td>
</tr>
</tbody>
</table>

Table 4.4. Summary of aggregation parameters.
Overall constants reflecting events at both ends; See corresponding Figures 6-8 for significance values.

<table>
<thead>
<tr>
<th>Tau Species</th>
<th>$K_{crit}^a ±$ SEE (µM)</th>
<th>$k_e^- a ±$ SEE (s⁻¹)</th>
<th>$k_e^+ a$ (mM⁻¹s⁻¹)</th>
<th>Lag time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified</td>
<td>0.29 ± 0.02</td>
<td>2.25E-2 ± 2.55E-3</td>
<td>77.78 ± 10.48</td>
<td>0.67 ± 0.03</td>
</tr>
<tr>
<td>5 mol/mol</td>
<td>0.83 ± 0.08</td>
<td>4.34E-2 ± 4.92E-3</td>
<td>52.11 ± 7.74</td>
<td>0.92 ± 0.07</td>
</tr>
</tbody>
</table>
4.6. Figures

![Modification Sites Diagram]

**Figure 4.1. Summary of modification sites identified by LC-MS/MS on immunopurified PHF tau and tau enriched from pathologically normal human brain.**

The sequence shown is that of human 2N4R tau (NCBI accession number NP_005901). Blue, PHF tau sequence coverage; Red, pathologically normal sequence coverage; Purple, shared sequence coverage; Underline, MTBR (as defined by Goedert et al., 1989); U, ubiquitylated sites; me1, monomethylated sites; me2, dimethylated sites; ReMe, sites modified by reductive methylation at high methylation stoichiometry (22 mol/mol); HEK, sites modified in tau-expressing HEK293 cells; identified by MS analysis reported herein and in (Cripps et al., 2006; Thomas et al., 2012).
Figure 4.2. Reductive methylation of tau protein as a function of time.

Tau was subjected to reductive methylation using conditions outlined in materials and methods. Reaction was monitored by $[^{14}C]$-labeled methyl, donated $[^{14}C]$-formaldehyde over a period of 60 minutes to calculate the mol methyl per mol tau stoichiometry. Data were fit to an exponential rise to max equation to estimate the methyl group incorporation. $n = 3$, mean ± SD.
Figure 4.3. Effect of methylation on tau-microtubule binding.
Paclitaxel-stabilized pre-formed microtubules were incubated with tau that was either unmodified or reductively methylated at 5 or 22 mol/mol methylation. Microtubules and bound tau were ultracentrifuged, the pellet separated by SDS-PAGE, and protein amount estimated by Coomassie blue staining optical density using appropriate protein standards. (A) Representative Coomassie-stained gel of unmodified tau. (B) Mean bound tau normalized to polymerized tubulin ± SD (n = 3) was plotted versus free tau and data fit to a four parameter logistic curve to calculate the Bmax (C), AC50 (D), and Hill slope (E) for each tau species. (●) Unmodified, (◊) 5 mol/mol (▲) 22 mol/mol. (C-E) Replot of parameters calculated from fitted curves in panel B ± SEE. No significant difference was found among Bmax or AC50 values. Both 5 and 22 mol/mol methyl incorporation depressed the Hill slope by half. *, p<0.05; n.s., p>0.05, as determined by z-test.
Figure 4.4. Effect of tau methylation on microtubule polymerization function.

Tubulin dimer was incubated with either no tau, unmodified tau or reductively methylated tau with varying degrees of methyl group incorporation. (A) Tubulin assembly was initiated by raising reaction temperature to 37°C and monitored by change in absorbance at 340 nm every minute for 60 minutes, alternating points of which are plotted against time for no tau (▼), unmodified (●), 5 mol/mol (▲), 10 mol/mol (■), 16 mol/mol (●), and 22 mol/mol (▲) methyl group incorporation. Data points represent 3 separate trials ± SD. Kinetics parameters were calculated from each of three trials by fitting data to an exponential rise to max to calculate extent and rate of polymerization. (B) Replot of data from Panel A, where each bar represents the extent of polymerization in absorbance units ± SD. High methylation stoichiometry (≥16 mol/mol) significantly depressed polymerization extent, relative to unmodified control. No significant difference was found between no tau control and very high methylation stoichiometry (22 mol/mol). ###, p<0.001 relative to no tau; ***, p<0.001 relative to unmodified tau, as determined by Bonferonni post hoc analysis.
Figure 4.5. Lys methylation decreases tau aggregation propensity. Unmodified (A) or reductively methylated tau: 5 mol/mol (B), 10 mol/mol (C), 16 mol/mol (D), or 22 mol/mol methylation (D) were incubated at 2 μM in the presence of Thiazine red inducer for 18 h at 37°C, then assayed for filament formation by electron microscopy. (A-E) Scale bar = 500 nm; Insets, Scale bar = 100 nm
Figure 4.6. Tau methylation increases aggregation critical concentration.

(A) Critical concentration of either unmodified (●) or 5 mol/mol methylated tau (○) were determined by assaying filament formation by transmission electron microscopy incubated at varying concentrations of tau in the presence of Thiazine red inducer for 18 hr at 37°C. Each data point represents total filament length as a function of bulk protein concentration (n = 3) ± SD, and solid lines represent best fit of the data points to linear regression. The abscissa intercept, which was obtained by extrapolation (dotted lines), was used to determine critical concentration ($K_{\text{crit}}$; see Table 4.4 for values). (B) Replot of data from Panel A, where each bar represents the $K_{\text{crit}}$ ± propagated SEE. Methylation increased $K_{\text{crit}}$ nearly 3-fold relative to unmodified tau. ***, p<0.001, as determined by z-test.
Figure 4.7. Rate constants for filament extension.

(A) Filaments prepared from unmodified (●) and 5 mol/mol methylated tau (○) in the presence of Thiazine red, and the resultant disaggregation was followed as a function of time by electron microscopy. Each data point represents total filament length per field ± SD (n = 3), whereas the solid line represents best fit of data points to an exponential decay function. The first-order decay constant $k_{\text{app}}$ was estimated from each regression and used in conjunction with filament length (shown in figure) and number at time $t = 0$ to calculate dissociate rate constant $k_c$. $k_{c+}$ was then obtained from the relationship $K_{\text{crit}} = \frac{k_c}{k_{c+}}$ (see Table 4.4). (B) Replot of data from Panel A and Table 4.4, where each bar represents the ratio of rate constants for filament extension ($k_{c+}$) and dissociation ($k_c$) determined for 5 mol/mol methylated versus unmodified tau ± propagated SEE. A ratio of 1, corresponding to no difference in rate, is marked by the dashed line. Methylation increased filament dissociation while decreasing filament extension. *, $p<0.05$; **, $p<0.01$ as determined by z-test for comparison of methylated versus unmodified rate constants.
Figure 4.8. Tau methylation depresses filament nucleation rate.

(A) Either unmodified (●) or 5 mol/mol methylated tau (○) were incubated at constant supersaturation (i.e., 0.3 μM above \( K_{\text{crit}} \)) in the presence of Thiazine red inducer, then assayed for filament formation as a function of time. Each data point represents average filament lengths/field calculated from triplicate electron microscopy images ± SD whereas normalized curve (solid lines) represents best fit of data to a three parameter Gompertz growth function (Necula and Kuret, 2004a). Values for lag time were estimated from these plots and are summarized in Table 4.4. (B) Replot of data from Panel A, where each bar represents the lag time ± propagated SEE. Methylation increased lag time relative to unmodified tau. ***, \( p<0.001 \), as determined by \( z \)-test.
Figure 4.9. Effect of tau methylation on the fibrillization pathway.
Normal tau binds tightly to microtubules. Methylation decreases tau affinity for microtubules, but is likely not relevant at physiological levels. Tau that is not bound to microtubules exists as a natively disordered, assembly-incompetent monomer ($U_x$). A conformational change to an assembly-competent state accelerates polymerization ($U_c$). Methylation may inhibit the assembly-competent monomer through stabilization of aggregation-resistant conformations. Once the assembly-competent species form, the rate-limiting step in tau fibrillation is formation of dimer, which represents the thermodynamic nucleus ($N$). Following nucleation, extension occurs through further addition of assembly-competent monomers to the filament ($F$) ends. Methylation inhibits filament formation through multiple points in the pathway with its greatest effect being the combination of decreased extension and increased disassembly of the filament.
Chapter 5: Conclusions and Future Directions

5.1. Introduction

Alzheimer’s disease (AD) is the most common form of dementia, affecting an estimated 5.4 million Americans and costing the US healthcare system about $200 billion annually (Alzheimer’s Association, 2012). AD is characterized initially by impaired memory, followed by changes in behavior and confusion and finally difficulty performing activities of daily living, such as walking and eating. Although diagnosis of AD is commonly made by a primary care physician on the basis of medical history and neurological evaluation, definitive diagnosis can only be made post mortem on the basis of pathology. However, recently the National Institute on Aging (NIA) and the Alzheimer’s Association recommended new diagnostic criteria that incorporate biomarker tests that would detect these lesions in living patients (Albert et al., 2011; Jack et al., 2011; McKhann et al., 2011; Sperling et al., 2011). The pathological lesions that define disease are senile plaques, composed of the amyloid beta (Aβ) peptide, and neurofibrillary tangles (NFTs), composed of the microtubule associated protein tau. Although the role these lesions play in disease progression is not well understood, they have a clear correlation to the disease progression, and thus, remain a starting point for studying disease.
The work herein describes the use of the traditional starting point of pathology to gain insight into the molecular mechanisms of disease progression. Although not one of the defining lesions, granulovacuolar degeneration (GVD) has a well documented relation to disease (Ball, 1977; Ball and Lo, 1977; Ghoshal et al., 2002; Lagalwar et al., 2007). However, because of the lack of a biochemical marker, the GVD field was eclipsed by the dawning of the molecular age in AD research with focus on tau- and Aβ-laden lesions. The ultrastructure of GVBs have been studied extensively and has characterized them as large (up to 5 μm) double-layered membrane-bound organelles harboring an electron dense granule (Okamoto et al., 1991). They are classically detected in AD tissue sections with histological stains, such as hematoxylin and eosin, but the discovery of casein kinase 1 delta (Ckiδ) as a robust, reliable marker of the lesions (Ghoshal et al., 1999; Kannanayakal et al., 2006) facilitated the in depth biochemical study described in chapter 2 (Funk et al., 2011). Because of their ultrastructural characteristics, a long standing hypothesis is that GVD bodies (GVBs) are organelles in the autophagic pathway of intracellular protein degradation via the lysosome (Okamoto et al., 1991). However, autophagic organelles are rarely seen in neurons due to their efficient clearance (Boland et al., 2008), therefore, we hypothesized that a defect in the autophagic pathway may be responsible for the accumulation of GVBs to the number and size seen in disease. In order to test this hypothesis, we co-immunolabeled sections of AD-affected brain tissue with anti-Ckiδ and antibodies directed against autophagic organelles at various stages of the pathway (Funk et al., 2011). Sections were imaged with laser scanning confocal fluorescence microscopy and relative colocalization of
autophagic markers with GVD marker, Ckiδ, was estimated. Results showed that GVD bodies resemble late stage autophagic organelles at the nexus of the autophagic and endocytic pathways. These data suggest a failure in the fusion of lysosomes with these late stage organelles. Furthermore, results indicate the endocytic pathway as an important contributor to lesion formation. We have proposed that this defect may contribute to several other features of AD, including the formation of senile plaques, neurofibrillary tangles, and tau detectable in the cerebrospinal fluid as a biomarker (see chapter 1).

Neurofibrillary lesions, composed of paired helical filaments (PHFs) of tau protein, have a well documented but incompletely understood connection to disease. Although certain genetic mutations in the gene that encodes tau, MAPT, are known to cause relatively rare cases of frontotemporal dementia (FTD), no such mutations cause AD (Rademakers et al., 2004). Rather, neurofibrillary lesion formation is accompanied by extensive post translational modification (PTM) to the tau protein (Martin et al., 2011). In order to determine the PTM signature most closely associated with NFT formation, we have begun mapping the tau modifications with single amino acid resolution using mass spectrometry methods (Thomas et al., 2012). PHF-tau was immunopurified from AD brain and subjected to nanoflow-liquid chromatography tandem mass spectrometry (LC-MS/MS). In addition to phosphorylation and ubiquitylation, the resulting spectra identified monomethylation as a previously unidentified modification (see chapter 3). The methyl-Lys was distributed among seven residues located in the projection domain and microtubule binding repeat region of the protein with one site, K254, being a substrate for either methylation or ubiquitylation. To
confirm that methylation is a wide-spread modification rather than a subset due to purification methods, tissue sections of AD-affected brain were subjected to double-label confocal fluorescence microscopy using anti-tau and anti-methyl-Lys antibodies. Results show that most NFTs co-labeled with anti-methyl-Lys antibody. Together, these data provide the first evidence that tau is post translationally modified by Lys methylation.

The most studied tau PTM is phosphorylation, which increases 3- to 4-fold in AD relative to non-diseased control (Ksiezak-Reding et al., 1992). In order to determine whether methylation undergoes similar changes in modification stoichiometry with disease onset, we extended analysis of the PTM signature to tau enriched from pathologically normal human brain tissue, again using LC-MS/MS (see chapter 4). Resulting spectra demonstrate that, contrary to phosphorylation, methylation decreases with disease onset. In order to determine the effect of tau methylation on the normal and pathological functions of tau, we prepared methylated tau \textit{in vitro} by reductive methylation. Although the critical \textit{in vivo} functions of tau are not well understood, the most studied function involves microtubule binding and stabilization (Feinstein and Wilson, 2005). Using biochemical methods to probe this accepted function of tau, we have determined that while methylation slightly decreases the tight binding of tau to microtubules, its ability to promote microtubule assembly is not hindered by low levels of methylation. We have further dissected the effect of methylation on the pathological role of tau in aggregation. Results show that even modest methylation drastically reduces the propensity of tau to aggregate into filaments, suggesting that the benefit of tau
methylation may be in its protection against self-association into pathological aggregates during normal aging.

The trigger of disease onset is not clear, but likely involves multiple factors rather than a single cause. Certain genetic mutations in the gene for the amyloid precursor protein (APP) as well as presenilin 1 and presenilin 2, which are involved in the proteolytic processing of APP into Aβ, are known causes of disease; however, these genetic mutations are responsible for less than 1% of AD cases (Alzheimer’s Association, 2012). The greatest risk factor of AD is age, but AD is not a normal part of aging. Other risk factors that likely contribute to risk of disease include genetics, cardiovascular health, diet, and head injury. One hypothesis is that these factors may increase risk for AD by improperly modulating one-carbon metabolism (Figure 5.1). For example, the vitamins folic acid, B6, and B12 serve as coenzymes in this metabolism, in which a carbon unit from serine or glycine reacts with tetrahydrofolate (THF) to form methylene-THF (Friso and Choi, 2005). This can be reduced to methyl-THF (MTHF) and used for the methylation of homocysteine (hcy) to form methionine, a proportion of which is converted to S-adenosylmethionine (SAM), the universal donor for methylation reactions (Janssens et al., 2008). A deficiency in these vitamins can result in diminished methionine and elevated hcy levels (Cantoni, 1985). In SAM-dependent methylation, S-adenosylhomocysteine (SAH) is produced as a byproduct of the reaction and is a potent competitive inhibitor of SAM-dependent methyltransferase activity (Chiang et al., 1977). In turn, SAH is converted to hcy by a reversible reaction catalyzed by SAH hydrolase (SAHH). Because the rate and direction of this reaction is governed by relative levels of
the metabolites, accumulation of hcy can lead to elevated SAH levels, and thus depress methyltransferase activity via competitive inhibition (Sontag et al., 2008). Vital SAM-dependent methylation is extensive in the brain, producing neurotransmitters, phospholipids, and myelin (Axelrod and Wurtman, 1966; Flynn et al., 1982; Kim et al., 1997). Methylation is also important in the regulation of presenilin expression, γ-secretase activity, and amyloid-β levels (Sontag et al., 2007; Sontag et al., 2008). Furthermore, the role of protein methylation on tau regulation is two-fold.

Tau is highly modified by post translational modifications (PTMs) including phosphorylation and methylation, among others (Martin et al., 2011). Perturbations in one-carbon metabolism are poised to play a dual role in modulating tau PTM status (Figure 5.1). First, although the methyltransferase responsible for tau methylation is unknown, it is most likely SAM-dependent. Thus, competitive inhibition of tau methyltransferases (TMTs) by SAH would result in decreased methylation stoichiometry. Second, tau phosphorylation state is mediated by the concerted activity of protein kinases, such as glycogen synthase kinase 3 beta (GSK3β), and protein phosphatases, principally protein phosphatase 2 A (PP2A) (Hanger et al., 2009; De-Paula et al., 2010). Carboxyl methylation of the PP2A catalytic subunit on L309 by SAM-dependent leucine carboxyl methyltransferase 1 (LCMT) enhances the biogenesis and stability of specific PP2A enzymes containing the Bα subunit, the primary PP2A isoform responsible for dephosphorylating tau (Sontag et al., 1996; Sontag et al., 1999; Sontag et al., 2008). Thus inhibition of LCMT by SAH would increase the phosphorylation state of tau by depressing the rate at which it is dephosphorylated. It has been shown that in AD, tau is
both hyperphosphorylated and hypomethylated. Thus, perturbation in one-carbon metabolism is a potential mechanism of dual regulation of these modifications.

Previous studies have demonstrated that tau phosphorylation varies with age and stage of development (Seubert et al., 1995). Similar to AD, tau isolated from fetal brain tissue is hyperphosphorylated; these levels decrease with maturation, then increase again with disease onset. Going forward, it will be important to determine the methylation status of tau as a function of development and aging. For this, we have acquired 12 samples of cortical brain tissue of varying age from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD (Table 5.1). The hypothesis outlined above would predict a parallel but opposite change in tau methylation with brain development and aging.

As discussed in chapter 4, methylation has a potent effect on both the normal and pathological functions of tau in vitro. Going forward, it will be important to identify the enzymes responsible for tau methylation and demethylation. This will facilitate functional studies of tau methylation in cells and direct new avenues of research in AD therapeutics. Although there are hundreds of known substrates of SAM-mediated methylation, methyltransferases are found in a small number of distinct structural arrangements used to classify them into superfamilies, each of which share conserved amino acid sequences (Martin and McMillan, 2002; Schubert et al., 2003; Kozbial and Mushgian, 2005; Petrossian and Clarke, 2009a; Petrossian and Clarke, 2009b). SET domain methyltransferases catalyze the methylation of protein Lys residues (Xiao et al., 2003; Dillon et al., 2005; Webb et al., 2008). Based on the profiles of primary sequences,
predicted secondary structures, and solved crystal structures of known methyltransferases, computational methods have predicted 208 proteins that make up the human methyltransferasome. This equates to ~1% of all human gene products, 57 of which are predicted to be SET methyltransferases containing supplemental domains that promote substrate binding specificity (Petrossian and Clarke, 2011).

Until recently it was believed that Lys methylation was an irreversible reaction. The discovery of Lys-specific demethylase-1 (LSD1) in 2004 suggested a new perspective on methylation dynamics (Shi et al., 2004; Metzger et al., 2005). LSD1 is a representative enzyme in a class of amine oxidases that catalyze Lys demethylation in a flavin adenine dinucleotide (FAD)-dependent manner (Shi et al., 2004). Members of this class of demethylases cannot act on trimethyl moieties owing to the absence of the protonated nitrogen required for oxidation (Shi et al., 2004; Metzger et al., 2005). Instead, removal of trimethyl moieties, in addition to mono- and di-methyl moieties, is achieved by a second class of demethylases comprising Jumonji-C (JmjC) domain-containing proteins (Klose et al., 2006). The demethylation reaction carried out by these proteins requires the presence of Fe(II) and α-ketoglutarate to generate formaldehyde and succinate. Because of the relative newness of the demethylase field, significantly less is known about them.

In order to focus on methyltransferases and demethylases most likely to act on tau protein, the NCBI Gene Expression Omnibus (GEO) data base was mined for Lys methyltransferases and demethylases that are highly expressed in both normal and AD brain at the mRNA level. Two relevant data sets were identified that analyze gene
expression within AD-relevant areas: GDS 2795 evaluates the mRNA expression of neurons containing neurofibrillary tangles and histopathologically normal neurons in the entorhinal cortex of 10 mid-stage AD patients that were separated using laser-capture microdissection methods (Dunckley et al., 2006), and GDS810 profiles mRNA expression of brain hippocampi from 24 post mortem subjects with AD at various stages of severity and 9 control samples (Blalock et al., 2004). To identify expressed methyltransferases, datasets were queried for “SET* OR EHMT* OR EZH*” keywords, resulting in 32 and 70 hits, respectively. To identify demethylases, datasets were queried for “KDM* OR JMJ*” keywords, resulting in 43 and 73 hits, respectively. The percentile rank within the test set (i.e., the relative expression level) was recorded for each sample, averaged for each gene, and sorted according to their mean ranks. Genes with a mean rank of ≥50 for either data set was included for further consideration, resulting in 20 unique methyltransferases and 23 unique demethylases. These lists were pruned to exclude those without known enzyme activity, resulting in 18 unique methyltransferases (Table 5.2) and 21 unique demethylases (Table 5.3). Future work will focus on further pruning these lists to a tractable number of promising candidates for cellular transfection experiments, discussed below.

With the identification of responsible tau methylation/demethylation enzymes, studies probing the functional outcomes of tau methylation will be feasible. Three avenues of study are of particular importance: cellular function, cytotoxicity, and PTM cross talk. First, in vitro studies have suggested that tau methylation inhibits microtubule binding and stabilization; however, this modest inhibition is likely of little significance.
considering the relatively low physiological levels of tau methylation and the high cellular concentrations of tau and tubulin. It is possible, though, that tau methylation affects cells in ways that have yet to be described. Second, the Kuret lab has developed a cellular model of tau aggregation using HEK-293 cells that express full length (2N4R) tau under a tetracycline-inducible promoter (TRex-293 cells) (Bandyopadhyay et al., 2007). We have shown that in this model, tau is methylated but at low levels (see chapter 4). By transfecting these cells with methyltransferase-encoding DNA to boost tau methylation status and treating them with the aggregation inducer congo red, cytotoxicity of tau aggregation, and potentially its alleviation by methylation, can be studied under physiological but tractable conditions. Third, we have demonstrated the potential for PTM cross talk by mass spectrometry methods (see chapters 3 and 4); however, these methods do not easily allow the study of the temporal relationships among these modifications. The ability to modulate tau methylation in cells by transfecting with methyltransferase and /or demethylase cDNA will facilitate the determination of PTM crosstalk (i.e., the effect of one modification on a neighboring modification). For example, tau phosphorylated by the microtubule affinity-regulating kinase 2 (PAR1/MARK2) is not recognized by the CHIP/Hsp90 E3 ligase complex and thus fails to be ubiquitylated or degraded (Dickey et al., 2008). Conversely, tau requires phosphorylation by GSK3b for efficient recognition and ubiquitylation by the CHIP/Hsp90 complex (Shimura et al., 2004). Considering that there is direct competition between ubiquitylation and methylation, it will be important to determine the result of this competition.
The results of these proposed studies will help identify potential points of therapeutic intervention. We have shown that tau methylation is protective against tau aggregation into pathological filaments. Identification of the enzymes responsible for the modification of tau would elucidate novel therapeutic targets to either increase methylation or decrease demethylation and inhibit tau aggregation into pathological filaments. Furthermore better understanding of the crosstalk among these modifications may reveal not only additional therapeutic targets but also a better understanding of the causes and consequences of changes in PTM status and its role in both health and disease. Thus, we anticipate that the findings of these studies will be broadly applicable to not only neurodegenerative diseases, such as AD, but also neurodevelopment.
5.2. Tables

Table 5.1. Case demographics.
Race: AA, African American; C, Caucasian; PMI, post mortem interval; COD, cause of death.

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<th>Race</th>
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**Table 5.2. Candidate Methyltransferases**

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5.3. Figures

Figure 5.1. Schematic model showing a hypothetical mechanism of systemic methylation/phosphorylation coordination.

Tau is modulated by both lysine methylation and hydroxyl-phosphorylation. Environmental factors, such as folate deficiency, may mediate these two modifications simultaneously by increasing Hcy levels. Hcy can be converted to SAH, the byproduct of SAM-mediated methyltransferases, and a potent inhibitor of methyltransferase activity. Two methyltransferases of importance are tau methyltransferases and LCMT, which activates PP2A, the phosphatase responsible for dephosphorylating tau protein. Thus the inhibition of SAM-mediated methyltransferases could result in the increased phosphorylation of tau through decreased phosphatase activity as well as reduced methylation, both of which promote tau aggregation and are consistent with the PTM signature seen in authentic disease. Abbreviations: SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; Hcy, homocysteine; Met, methionine; MTHF, methyltetrahydrofolate; THF, tetrahydrofolate; COMT, catechol-O-methyltransferase; SAHH, SAH hydrolases; MTR, methionine synthase; LCMT1, leucine carboxymethyltransferase; PP2A, protein phosphatase 2A; GSK3β, glycogen synthase kinase 3 beta; TMT, Tau methyltransferase; TDM, Tau demethylase; P-tau, phospho-tau; Me-tau, methyl-tau

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References


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