Lysine Methylation is an Endogenous Post-Translational Modification of Tau Protein in Human Brain and a Modulator of Aggregation Propensity

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Introduction

Alzheimer’s Disease (AD) is the most common form of dementia, affecting 13% of Americans aged at least 65 years and 43% of Americans aged at least 85 years. It is a major driver of long-term care costs in the U.S., and there is currently no cure or preventative treatment. AD is defined by the appearance of two hallmark lesions: extracellular plaques composed of the beta-amyloid peptide and intracellular neurofibrillary tangles (NFTs) composed of the microtubule-binding protein tau. The accumulation of these proteins in aggregated forms is believed to contribute to the pathogenesis of AD. However, the accumulation of NFTs better correlates with disease and symptom progression, and the quantity and distribution of NFTs, not plaques, are used to definitively diagnose AD post mortem. For these reasons, the biology of normal tau protein, and how it malfunctions in disease, is being investigated for clues to the mechanisms underlying AD pathogenesis. Normal tau protein functions in monomeric form to stabilize microtubules and promote their assembly. In AD, however, tau aggregates into long fibrils that eventually grow to fill the neuronal cytoplasm. Candidate triggers for this conformational change include post translational modifications (PTMs). Previous work identified phosphorylation of Ser and Thr residues as major physiological PTMs capable of triggering tau aggregation. More recent work conducted in model systems has revealed that
PTMs of Lys residues can also serve to modulate tau aggregation propensity. But to date, Lys modifications have not been demonstrated at single residue resolution in authentic human brain specimens.

To test the hypothesis that tau is normally modified on Lys residues, tau was isolated from normal human brain (n = 4; all cases 55 yr old) and subjected to a “bottom up” proteomic characterization. In this approach, purified human tau protein was digested with the proteolytic enzyme trypsin, and the resulting peptides characterized by mass spectroscopy methods. The resulting structural characterization revealed methylation as a novel tau PTM on Lys residues. The modification clustered predominantly in the C-terminal region of the tau molecule known to mediate pathological self association. To characterize the effects of Lys methylation on tau function, purified recombinant tau was subjected to chemical methylation to generate a library of modified tau species that varied with respect to methylation stoichiometry. The methylated samples were then tested for their ability to promote microtubule assembly (i.e., normal tau function), as well as their ability to aggregate (i.e., pathological malfunction). To test the effect of methylation on tau’s microtubule assembly promoting activity, methyl tau was incubated with tubulin and an absorbance reading was taken every two minutes over the course of an hour to monitor the formation of microtubules. This experiment revealed that at lower, physiological levels, methylation did not inhibit tau’s ability to promote microtubule assembly. In contrast, a transmission electron microscope study examining aggregate formation of methyl tau showed that even low, physiological levels of methylation were able to inhibit tau aggregation. To characterize the mechanism of inhibition, the effects of Lys methylation on tau aggregation propensity was examined as function of time and tau protein concentration. These studies revealed that methylation inhibited aggregation by increasing the amount of protein needed to
form aggregates. It did so by increasing tau’s dissociation rate from fibrils, and decreasing the fibril extension rate. In addition, methylation slowed aggregation rate by depressing filament nucleation, the rate limiting step in tau filament formation.

Together these data show that low-occupancy methylation is a normal human tau PTM that does not affect tau’s normal function, but that does depress tau aggregation propensity. The experimental findings point to methylation being a potential regulatory PTM of tau. We postulate that methylation may decrease in AD compared to normal brain, and that maintaining or increasing methylation of tau could be a therapeutic approach for slowing the rate and extent of neurofibrillar lesion formation in AD.

Results

The first part of our study focused on identifying PTM in normal human brain. Soluble was isolated from brain samples of cognitively normal human males aged 55 ± 1.2 years.

<table>
<thead>
<tr>
<th>Case (#)</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Race</th>
<th>PMI (h)</th>
<th>Source</th>
<th>History</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>54</td>
<td>M</td>
<td>C</td>
<td>---</td>
<td>OSU</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td>54</td>
<td>M</td>
<td>AA</td>
<td>10</td>
<td>NICHD</td>
<td>HASCVD; PTSS, No history of drug, alcohol abuse</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>M</td>
<td>AA</td>
<td>13</td>
<td>NICHD</td>
<td>HASCVD; History of Diabetes, cocaine abuse, and hypertension</td>
</tr>
<tr>
<td>4</td>
<td>57</td>
<td>M</td>
<td>C</td>
<td>5</td>
<td>NICHD</td>
<td>Acute Bronchopneumonia/ASCVD; history of alcohol abuse</td>
</tr>
</tbody>
</table>

Table 1. Case Demographics.

AA, African American; C, Caucasian.

Post-mortem interval.

OSU, The Ohio State University Buckeye Brain Bank; NICHD, NICHD Brain and Tissue Bank for Developmental Disorders.

ASCVD, atherosclerotic cardiovascular disease; HASCVD, hypertensive arteriosclerotic cardiovascular disease; PTSS, Post Traumatic Stress Syndrome.
These samples displayed no pathological plaque and tangle formation. These samples were subjected to trypsinization and then LC-MS/MS. The mass spectrometry data was investigated using SEQUEST and MASCOT softwares to identify post translational modifications. The data revealed a number of previously identified phosphorylation sites, as well as novel mono- and di- methylation sites on Lys residues. The peptides investigated covered 90% of the sequence of the longest tau isoform 2N4R (shown in Figure 1), as well as 90% of Ser/Thr residues and 77% of Lys residues. (Fig.1). The phosphorylation sites predominantly corresponded with previously known sites. However, methylation sites were novel, identifying methylation as a normal PTM in human brain. The methylation primarily occurred in the microtubule binding repeat region of tau, responsible for both normal tubulin assembly and pathological aggregation. Due to the importance of this region, we further investigated what effects methylation may have on both normal tau function and pathological aggregation.
FIGURE 1. Summary of modification sites on human tau proteins identified by LC-MS/MS.

A. Identified tryptic peptides in the context of the human 2N4R isoform (NCBI accession number NP_005901), where the dotted underline depicts projection domain segments n1 and n2, the solid underline identifies the MTBR (as defined by (69)), and PHF6/PHF6* mark the hexapeptide segments involved in filament nucleation. Font color depicts sequence coverage: blue, identified only in tissue-derived tau, red, identified only in recombinant 2N4R tau containing near-saturating levels of methylation (21.8 mol/mol), and purple, identified in both. Phosphorylation (P) sites are marked by green circles (hollow circles, novel sites; solid circles, previously reported sites), whereas methylation sites are marked by orange (me1, monomethylation) and red (me2, dimethylation) symbols (squares for tissue tau, triangles for recombinant tau).

B. Tau methylation and phosphorylation site distribution map (2N4R tau), showing location of modification sites relative to projection domain segments n1 and n2 and MTBR repeats r1 – r4. The length of each bar corresponds to the number of cases in which the modification was found.
First we explored tau’s ability to perform its normal function, microtubule assembly. Recombinant 2N4R human tau was reductively methylated in vitro to varying degrees to investigate the role of methylation on microtubule assembly. Methylation was performed by incubating 2N4R tau with formaldehyde and sodium cyanoborohydride at room temperature at variable time up to one hour. Methylation stoichiometry was determined by performing the reductive methylation using C14 formaldehyde. Methylation time course was modeled as a simple exponential growth to maximum using the function:

$$y_t = y_{\text{max}}(1 - e^{-k_{\text{app}}t})$$  \hspace{1cm} (1)

where $k_{\text{app}}$ and $y_{\text{max}}$ are the rate constant and maximum extent of methylation, respectively. The methylation stoichiometries and time course are seen in Figure 2A.

To determine the effect of methylation on tau-promoted microtubule assembly, purified tubulin dimers were incubated at 37°C alone or with unmodified or reductively methylated tau, and the extent of tubulin polymerization measured spectrophotometrically. When tau was methylated at low levels (6 mol/mol and 10 mol/mol) tubulin polymerization was no different than the non methylated control (Fig 2BC). However, higher levels of methylation (16 mol/mol and 22 mol/mol) inhibited tau’s ability to promote microtubule assembly (Fig 2BC). Overall, the data shows that only very high levels of methylation inhibit tubulin polymerization, and that low levels do not change tau’s ability to perform its normal function.
Additionally, we investigated the effect of methylation on tau’s pathological function of aggregation. Reductively methylated tau of varying stoichiometries was incubated with thiazine red aggregation inducer under physiological pH, reducing conditions, and ionic strength. These aggregated samples were observed using transmission electron microscopy (TEM). The non-methylated tau sample displayed aggregates similar to authentic brain-derived tau (Figure 3A). However, low levels of methylation severely inhibited tau aggregation. Figure 3B shows that even at the lowest methylation stoichiometry, 6 mol/mol, aggregation propensity is inhibited greatly. Although the amount of aggregation was diminished, the morphology of the filaments that did form were the same among all the samples, methylated and unmodified. This reveals that methylated tau shared the same aggregation characteristics as unmodified tau, but has a lower propensity for aggregation.
To extend our study, we investigated the mechanism through which methylation inhibits aggregation. We used the unmodified control, and the lowest methylated sample (6 mol/mol) since the 6 mol/mol sample still exhibited measurable aggregation. These samples were incubated with thiazine red inducer under the conditions described previously. The abscissa intercepts of tau concentration dependence plots revealed that the minimal concentration for aggregation of methylated tau (5.5 mol/mol methylation stoichiometry) increased nearly 3-fold relative to unmodified tau (Fig. 4AB). These data indicate that Lys methylation depressed aggregation propensity in part by increasing the concentration of tau needed to support fibril formation.

In nucleation-elongation reactions, such as the tau aggregation reaction the minimal concentration approximates $K_{\text{crit}}$, which corresponds to the equilibrium dissociation constant for elongation, $K_e$.

$$K_{\text{crit}} = \frac{k_e}{k_e^+}$$ (2)
$K_{\text{crit}}$ therefore reflects contributions from both the dissociation ($k_{e-}$) and association ($k_{e+}$) rate constants for filament elongation. As a result, changes in $K_{\text{crit}}$ may stem from changes in either filament stability ($k_{e-}$), efficiency of monomer association with filament ends ($k_{e+}$), or both. To differentiate among these possibilities, initial filament disaggregation rates were estimated for unmodified and methylated (5.5 mol/mol stoichiometry) tau by diluting preassembled filaments below the $K_{\text{crit}}$ and measuring filament length as a function of time. Under these conditions, loss of total filament length was found to decay following first-order kinetics (Fig. 4C). Rate constant $k_{e+}$ was then calculated from estimates of $k_{e-}$ and $K_{\text{crit}}$ using Eq. 2. Tau methylation was found increased $k_{e-}$ nearly 2-fold and decreased $k_{e+}$ ~1.5-fold (Fig. 4D), suggesting that methylation increased minimal concentration by both weakening filament stability and slowing the rate of elongation.

**FIGURE 4.** Lys methylation modulates tau filament extension rates. A, Unmodified (solid circle) or 6 mol/mol methylated tau (hollow circle) were incubated (18 h at 37°C) at varying bulk concentrations in the presence of 100 µM Thiazine red inducer, then assayed for filament formation by electron microscopy. Total filament lengths were then plotted against bulk protein concentration. B, Replot of data from panel A, where each bar represents the $K_{\text{crit}}$ ± propagated SEE C, Filaments prepared from unmodified (solid circle) and 5 mol/mol methylated tau (hollow circle) as described above were diluted below $K_{\text{crit}}$ in assembly buffer, and the resultant disaggregation followed as a function of time by electron microscopy. D, Replot of data from panel C, where each bar represents the ratio of rate constants for filament extension ($k_{e+}$) and dissociation ($k_{e-}$) 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.
In a homogeneous nucleation dependent reaction, the rate limiting step involves formation of a thermodynamic nucleus, defined as the least stable species reversibly interconverting with monomer. After a nucleus is formed, elongation proceeds efficiently. Overall aggregation rate depends on nucleation rate as well as protein concentration and the rate of elongation ($k_e$ and $k_{e+}$). To determine whether methylation affected nucleation rate, tau aggregation time course was quantified for both unmodified and methylated (5.5 mol/mol stoichiometry) tau at constant supersaturation in the presence of thiazine red inducer. Under these conditions, differences in reaction rate primarily reflect differences in rates of nucleation and protein concentration. When quantified over 24 hours, both time series displayed lag, exponential growth, and equilibrium phases (Fig. 5A). Reaction lag times, which vary inversely with nucleation rate, were then estimated by fitting data with a 3-parameter Gompertz growth function. Under these conditions, methylated tau aggregated with a significantly longer lag time relative to unmodified tau (Fig. 5B). These data show that Lys methylation slows the nucleation phase of the tau aggregation reaction. Overall, multi-site Lys methylation at 5.5 mol/mol can depress tau aggregation at both the nucleation and elongation steps without affecting microtubule polymerizing activity.

FIGURE 5. Lys methylation depresses tau filament nucleation rate. A, Either unmodified (solid circle) or 5 mol/mol methylated tau (hollow circle) was incubated at constant supersaturation (i.e., 0.3 µM above $K_{crit}$) in the presence of Thiazine red inducer, then assayed for filament formation as a function of time. Values for lag time were estimated from these plots. B, Replot of lag times determined from data in panel A, where each bar represents the lag time ± propagated SEE.
Summary

Figure 6 summarizes the findings of the previous studies. As is shown in figure 6, tau normally binds, stabilizes, and promoted tubulin assembly. In AD, tau dissociated from the microtubules, and undergoes a conformational change that makes it aggregation competent. These aggregation competent proteins are able to form a nucleus and then further aggregate into fibrils characteristic of AD. Our data shows that tau is a normally methylated in human brain samples. Methylation at low stoichiometries does not inhibit tau’s normal function, but it does modulate aggregation propensity. Methylation strongly inhibits fibrilization by decreasing nucleation rate, decreasing fibril extension rate, and increasing dissociation rate. This data suggests that methylation could be a potential therapeutic target for AD. Increasing or maintaining the levels of methylation of tau could prevent or slow fibril formation. Further studies are needed to examine the methylation status of AD patients, as well as to explore avenues through which methylation levels of tau are controlled.

FIGURE 6. The effect of methylation on tau aggregation pathway.