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The Abundance of Nonphosphorylated Tau in Mouse and Human Tauopathy Brains Revealed by the Use of Phos-Tag Method

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Tauopathies are neurodegenerative diseases characterized by aggregates of hyperphosphorylated tau. Previous studies have identified many disease-related phosphorylation sites on tau. However, it is not understood how tau is hyperphosphorylated and what extent these sites are phosphorylated in both diseased and normal brains. Most previous studies have used phospho-specific antibodies to analyze tau phosphorylation. These results are useful but do not provide information about nonphosphorylated tau. Here, we applied the method of Phos-tag SDS-PAGE, in which phosphorylated tau was separated from nonphosphorylated tau *in vivo*. Among heterogeneously phosphorylated tau species in adult mouse brains, the nonphosphorylated ON4R isoform was detected most abundantly. In contrast, perinatal tau and tau in cold water-stressed mice were all phosphorylated with a similar extent of phosphorylation. In normal elderly human brains, nonphosphorylated ON3R and ON4R tau were most abundant. A slightly higher phosphorylation of tau, which may represent the early step of hyperphosphorylation, was increased in Alzheimer disease patients at Braak stage V. Tau with this phosphorylation state was pelleted by centrifugation, and sarkosyl-soluble tau in either Alzheimer disease or corticobasal degeneration brains showed phosphorylation profiles similar to tau in normal human brain, suggesting that hyperphosphorylation occurs in aggregated tau. These results indicate that tau molecules are present in multiple phosphorylation states *in vivo*, and nonphosphorylated forms are highly expressed among them. (*Am J Pathol* 2016, 186: 398–409; <http://dx.doi.org/10.1016/j.ajpath.2015.10.009>)

Alzheimer disease (AD) is the most common form of dementia in the elderly and is characterized by senile plaques composed of amyloid β peptides and neurofibrillary tangles (NFTs) that consist of hyperphosphorylated forms of tau protein.^{1,2} NFTs are also found in many other neurodegenerative diseases, including corticobasal degeneration (CBD), progressive supranuclear palsy, Pick disease, and frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). These diseases are collectively referred to as tauopathies.³ Because FTDP-17 is an inherited disease with mutations in the tau gene *MAPT*,^{4–6} mutation of tau is sufficient to cause neurodegeneration and dementia. Most mutations are found in the microtubule (MT)-binding

region of tau, but it is not known why mutations induce diseases. Tau in NFTs is hyperphosphorylated. Previous studies showed that hyperphosphorylated tau has a reduced MT-binding ability and a propensity to self-aggregate into paired helical filaments, a component of NFTs,^{7,8} but how tau becomes hyperphosphorylated during AD progression is not known. More than 40 phosphorylation sites are found in NFT-associated tau, with phosphorylation at Ser/Thr-Pro

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sequences being characteristic.^{9,10} It is still unclear whether there is disease type-specific phosphorylation of tau. It is also important to analyze tau phosphorylation more quantitatively and combinatorially to further understand the pathologic mechanism(s) of tauopathies.

Tau is a MT-associated protein, whose function is principally to polymerize and stabilize MTs in axons.¹¹ Tau consists of the N-terminal projection and C-terminal MT-binding domains. There are isoforms of tau with three or four MT-binding repeats, 3R or 4R, respectively, in the MT-binding domain, which are generated by alternative splicing of exon 10.¹² In addition, another alternative splicing in the N-terminal two insertions (exons 2 and 3) produces three isoforms, resulting in a total of six isoforms of tau. Expression of isoforms is regulated developmentally; 3R tau is expressed in fetal to neonatal mouse brain, and 4R tau is expressed in adult mouse brain.^{13,14} In adult human brain, all six isoforms are expressed.¹⁵ Although 4R tau has a stronger MT-binding ability than 3R tau, it is not known whether these isoforms have different functions *in vivo*.¹⁶ Tau is also phosphorylated physiologically.¹⁷ This phosphorylation regulates the MT-assembling activity of tau in a site-specific manner; phosphorylation in the MT-binding repeats abolishes the MT-binding activity, whereas that in the flanking regions only partially reduces this activity.^{16,18} Many kinases, including proline-directed protein kinases such as glycogen synthase kinase 3 β or cyclin-dependent kinase 5, phosphorylate tau at multiple sites both commonly and distinctly,¹⁰ but the precise role of site-specific phosphorylation is not yet understood.

Both normal and abnormal phosphorylation of tau were studied with the use of phospho-specific antibodies. Currently, many phospho-specific tau antibodies are commercially available. Although these antibodies are powerful and sensitive enough to detect a small change in phosphorylation at a particular site,¹⁹ it is difficult to determine the absolute ratio of phosphorylation and to make quantitative comparisons between experiments. In addition, many site-specific antibodies are generated to produce a complete phosphorylation profile. This is time consuming and expensive, and there are sometimes problems with specificity because of closely located phosphorylation sites. Thus, another simple and quantitative method for analysis of phosphorylated tau is needed.²⁰

Phos-tag SDS-PAGE is an established method that shifts the mobility of all phosphorylated proteins, depending on their number and sites of phosphorylation.²¹ We have previously used this method for quantitative measurement of combinatory phosphorylation of the p35 cyclin-dependent kinase 5 activator *in vivo*.²² In this study, we characterized phospho-tau species in brains of wild-type and AD model mice and in brains of human AD and CBD patients with the use of Phos-tag SDS-PAGE. The analyses revealed a number of interesting insights about *in vivo* phosphorylation of tau, indicating the usefulness of this method for studying tau phosphorylation.

Materials and Methods

Antibodies and Chemicals

Anti-Tau Ab-2 (Tau5), anti-phospho-Tau AT8, and anti-human tau HT7 were purchased from Thermo Fisher Scientific (Fremont, CA). Anti-unphospho-Tau (Tau-1), RD3, and RD4 were obtained from Millipore (Darmstadt, Germany). Phospho-Ser404 (P-S404) was obtained from Abcam (Cambridge, UK). Tau-C, the antibody against the C-terminal sequence of tau (amino acids 422 to 438), was described previously.²³ Anti-4R was described previously.²⁴ 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), Phos-tag acrylamide, microcystin LR, and alkaline phosphatase were purchased from Wako Chemicals (Osaka, Japan). Leupeptin was obtained from the Peptide Institute (Osaka, Japan).

Expression and Purification of Tau

Recombinant human tau, 0N3R, 0N4R, 1N3R, 1N4R, 2N3R, and 2N4R, and mouse tau, 0N3R, 0N4R, and 1N4R, were synthesized in *Escherichia coli* cultures and were purified as described previously.²⁵ Protein concentration was determined by Coomassie brilliant blue staining of the polyacrylamide gels with the use of bovine serum albumin as a standard.

Preparation of Extracts from Mouse and Human Brains

All animal studies were conducted in compliance with the guidelines for animal experimentation of Tokyo Metropolitan University and the Tokyo Metropolitan Institute of Medical Science. The P301L tau transgenic mouse line (JNPL3)²⁶ was obtained from Taconic (Hudson, NY).²⁷ The 5 $\times$ FAD mice²⁸ that have transgenes of human amyloid precursor protein and presenilin-1 with five mutations found in familial AD were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in cages of two to five littermates with access to food and water *ad libitum* in an environment subjected to a 12-hour light/dark cycle in the animal facility of the Tokyo Metropolitan Institute of Medical Science. C57BL/6J mice were obtained from Sankyo Labo Service (Tokyo, Japan). C57BL/6J mice at 96 weeks of age (96W) were provided by the Tokyo Metropolitan Institute of Gerontology. The cerebral cortices from mouse brains were individually homogenized in 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EGTA, 0.8 mol/L NaCl, 10% sucrose, 10 mmol/L NaF, 10 mmol/L β -glycerophosphate, 0.4 mmol/L AEBSF, 10 μ g/mL leupeptin, and 1 mmol/L dithiothreitol. After centrifugation at 15,000 $\times$ g for 20 minutes, the supernatant fluid was used as the brain extract. Sarkosyl-insoluble and -soluble fractions were separated as described.²⁹

Human brain tissues were obtained from the brain bank of the Tokyo Metropolitan Institute of Gerontology, and the study that used human brain tissues was approved by the

Table 1 Summary of Cases

Case	Age, years	Sex	PMI, hours	NFT, Braak stage
a	77	F	17.1	I
b	76	F	20.5	I
c	75	M	31.5	II
d	75	F	32.3	I
e	77	F	9.0	I
f	76	M	3.8	II
g	79	F	3.4	V
h	80	F	6.7	V
i	77	M	11.4	V
j	76	M	15.8	VI
k	76	M	15.8	VI
l	77	F	6.7	VI
m	79	M	12.3	II
n	74	F	53.5	I
o	69	F	3.3	I

F, female; M, male; NFT, neurofibrillary tangle; PMI, postmortem interval.

ethics committees of Tokyo Metropolitan University and the Tokyo Metropolitan Institute of Gerontology. Detailed descriptions of the subjects are given in Table 1. Braak stages were determined as described.^{30,31} The brain extracts were prepared from blocks of frozen human brains, consisting of the temporal cortical and precentral gyrus regions, by homogenizing in 10 mmol/L Tris-HCl (pH 7.4), 10 mmol/L NaF, 10 mmol/L β-glycerophosphate, 0.4 mmol/L AEBSF, 10 μg/mL leupeptin, and 1 mmol/L dithiothreitol. Sarkosyl-insoluble and -soluble fractions were prepared as described.²⁹

Treatment of Mice with Ice-Cold Water

Mice were immersed up to the neck in ice-cold water for 5 minutes. Then, they were gently wiped dry and returned to their cages at room temperature.^{32,33} After 20 and 90 minutes, the mice were sacrificed by cervical dislocation, and the brains were dissected.

Laemmli SDS-PAGE, Phos-Tag SDS-PAGE, Immunoblotting, and Densitometric Scans

Laemmli SDS-PAGE was performed with the use of 12.5% (w/v) polyacrylamide gels, and Phos-tag SDS-PAGE was performed with the use of 7.5% (w/v) polyacrylamide gels that contained 50 μmol/L Phos-tag acrylamide and 150 μmol/L MnCl₂ as described.^{22,25,34} For immunoblotting, proteins separated in a gel were transferred to a polyvinylidene difluoride membrane with the use of a submerged blotting apparatus, and immunoreaction was visualized by exposure on X-ray film (Fuji Film, Tokyo, Japan) with the use of the Enhanced Chemiluminescence Detection kit (GE Healthcare, Tokyo, Japan) as described previously.²⁵ The band intensity and banding profiles were analyzed with ImageJ version 64 software (NIH, Bethesda,

MD; <http://imagej.nih.gov/ij>) after densitometric scanning of X-ray films.

Results

Phosphorylation States of Tau in Mouse Brain Analyzed by Phos-Tag SDS-PAGE

We analyzed phosphorylation of tau in mouse brains by immunoblotting with a phosphorylation-independent antibody against Tau (Tau5) after Phos-tag SDS-PAGE. Tau in adult mice at 10W separated into more than four bands with a smear background in Phos-tag SDS-PAGE (Figure 1A). The electrophoretic mobility was compared with recombinant mouse tau 0N3R, 0N4R, and 1N4R. The lowest strong band migrated slightly faster than recombinant 0N4R tau. To see which tau bands were phosphorylated, we treated the brain extract with alkaline phosphatase and ran it in parallel with an untreated sample on Phos-Tag SDS-PAGE (Figure 1B). The mobility of the lowest band was not changed by this treatment, but the upper bands shifted down to the second band from the bottom, which migrated slightly faster than recombinant 1N4R tau. Given that the 4R tau isoforms 0N and 1N are main isoforms expressed in adult mouse,^{13,14} these two fastest migrating tau bands in the alkaline phosphatase-treated sample likely correspond to nonphosphorylated 0N4R and 1N4R tau. However, most of the upper bands in the untreated extract were phosphorylated forms of tau appeared at the second band from the bottom. Their densitometric scan profiles are also shown (Figure 1). The lowest band accounted for approximately 15.5% ± 1.5% (n = 3) of total tau in the brain extract before the alkaline phosphatase treatment. These results indicate that tau isoforms are differentially phosphorylated; the 0N4R isoform is primarily nonphosphorylated, and 1N4R tau is more phosphorylated in mouse brain. The phosphorylation of tau changes with brain development.^{35–38} We examined the phosphorylation status of tau in mouse brains at postnatal day 2 (P2) and 96W in addition to the 10W brains with the use of Phos-tag SDS-PAGE. Tau at P2 showed considerably slower migration than tau at 10W in Phos-tag SDS-PAGE immunoblotting (Figure 1C), confirming higher phosphorylation of tau in early postnatal days. In contrast, the banding pattern of tau in older mice at 96W was similar to that at 10W (Figure 1C). The main isoforms of tau expressed in brain change from 3R to 4R at around P20.^{14,39} To confirm that the difference in the phosphorylation state between P2 and 10W tau is not because of a difference in isoforms (ie, 3R versus 4R), we performed immunoblots with 3R- and 4R-specific antibodies, RD3 and RD4, respectively (Figure 1C).^{40,41} The results were consistent with previous reports that the 3R isoform is expressed during perinatal days and that 4R is expressed in adult mouse brains.^{13,14} Longer exposure of the blot for RD3 revealed the existence of a small amount of 3R tau in brain at 10W with migration similar to 4R tau (Figure 1C), indicating that, like 4R tau, 3R tau is less phosphorylated in adult brain.

Figure 1 Phosphorylation states of tau in mouse brain analyzed by Phos-tag SDS-PAGE. **A:** An immunoblot of tau in adult mouse brain with the use of Phos-tag SDS-PAGE. A brain extract was prepared from a mouse at 10W and was immunoblotted with Tau5 after Phos-tag SDS-PAGE. Mouse recombinant tau isoforms 0N3R, 0N4R, and 1N4R are shown in the **left lane** (r-mtau). **B:** Dephosphorylation of mouse tau with ALP. The mouse brain extract was incubated with 0.6 U/ μ L ALP for 6 hours at 37°C and immunoblotted with Tau5 after Phos-tag SDS-PAGE. The **left lane** is the brain extract before ALP treatment (—). Densitometric scans of control and dephosphorylated tau are shown at the right, black for control and gray for dephosphorylated tau. **C:** Age-dependent isoform expression and phosphorylation of tau in mouse brains. Brain extracts were prepared from mice at ages of P2, 10W, and 96W. Phosphorylation of tau was assessed by immunoblotting with Tau5, RD3, and RD4 after Laemmli (**upper panels**) or Phos-tag SDS-PAGE (**lower panels**). The **right panel** of RD3 is a longer exposure of the second lane (10W long). **D:** *In vitro* phosphorylation and dephosphorylation of tau in the brain extracts examined with the use of Phos-tag SDS-PAGE. Brain extracts were incubated at 37°C for the indicated times in the absence (—) or presence of ATP and MC and blotted with Tau5 after Phos-tag SDS-PAGE (**lower panels**). **Upper panels** are Laemmli SDS-PAGE. **E:** *In vitro* phosphorylation of recombinant 1N3R and 1N4R tau in the P2 and 96W brain extracts were examined with Phos-tag SDS-PAGE. Recombinant tau was incubated with brain extracts at 37°C for the indicated times in the presence of ATP and MC and blotted with anti-human tau HT7 after Phos-tag SDS-PAGE. HT7-reactive non-tau band is indicated by **arrow** in lane of time 0 in the adult brain extract. ALP, alkaline phosphatase; ATP, adenosine triphosphate; Lae, Laemmli SDS-PAGE; MC, microcystin LR; Phos, Phos-tag SDS-PAGE; P2, postnatal day 2; RD3, anti-3R tau; RD4, anti-4R tau; 10W, 10 weeks of age; 96W, 96 weeks of age.

Changes in tau phosphorylation are caused by perturbation of the balance between tau kinases and phosphatases. Previous results suggest that the higher kinase activity occurs during the neonatal period.^{38,42} We also tested which of the kinases or phosphatases are the main determinants in phosphorylation of perinatal and adult tau by incubating the brain extracts in the absence or presence of adenosine triphosphate and microcystin LR phosphatase inhibitor. Although a mobility change could be detected in Laemmli SDS-PAGE, the shifts were large enough in Phos-tag SDS-PAGE that dephosphorylation (**Figure 1D**) and phosphorylation (**Figure 1D**) could be tracked easily. The speed and extent of dephosphorylation were higher in the 10W and 96W extracts than in the P2 extract. Moreover, tau shifted upward to a greater extent in the 10W and 96W brain extracts than in the P2 extract in the presence of adenosine triphosphate and microcystin LR, implying that kinase activity is stronger in adult brains. However, it is also

possible that adult 4-repeats (4R) tau is more prone to being phosphorylated than perinatal 3-repeats (3R) tau. To investigate this possibility, we phosphorylated recombinant human 3R and 4R tau in the P2 or 96W brain extracts in the presence of adenosine triphosphate and microcystin LR (**Figure 1E**). To detect human tau specifically, we used anti-human tau antibody HT7. Although HT7 did not react with endogenous mouse tau, there was a HT7 reactive band with higher molecular weight only in the 96W extract (**Figure 1E**), which was also phosphorylated by incubation with adenosine triphosphate and microcystin LR. When the phosphorylation was assessed by the upward shift, interestingly it was found that 3R and 4R tau were differently phosphorylated; 4R tau was phosphorylated faster and more than 3R tau in the P2 and 96W extracts. Furthermore, the 96W extract showed similar or slightly higher phosphorylating activity to both 3R and 4R tau. Taken together, these results suggest that lower phosphorylation of tau in

Figure 2 Phosphorylation of tau in cold water–treated mice examined by Phos-tag SDS-PAGE. Mice at 6 weeks of age were treated with ice-cold water for 5 minutes and allowed to recover for 20 or 90 minutes at room temperature. Their brains were dissected, and tau was immunoblotted with Tau5, AT8, and Tau1 after Laemmli SDS-PAGE (**upper three panels**) or with Tau5 after Phos-tag SDS-PAGE (**bottom panel**). The control extract from a mouse brain collected without cold-water treatment is shown in the **left lane**. Lae, Laemmli SDS-PAGE; Phos, Phos-tag SDS-PAGE.

adult brain is because of higher protein phosphatase activity but not kinase activity.^{43–45}

Phosphorylation of Tau in Mice Exposed to Cold-Water Stress

Cold-water stress induces hyperphosphorylation of tau by reducing phosphatase activity.^{32,33,46} With the use of Phos-tag SDS-PAGE, we examined hyperphosphorylation of tau induced in mice by exposure to cold water for a 5-minute period, followed by varying recovery times of 20 and 90 minutes. The increased phosphorylation was confirmed at Ser199/Ser202/Thr205 by immunoblotting with AT8 (this antibody detects phosphorylated tau) and Tau1 (this antibody detects unphosphorylated tau) (Figure 2). The effect of the cold-water treatment was clearly observed by an upward shift of overall tau species in Phos-tag SDS-PAGE on detection with Tau5 (Figure 2). The lowest nonphosphorylated band disappeared, and upper phosphorylated bands increased at 20 minutes of recovery after the cold-water treatment. After 90 minutes, the tau bands mostly returned to the level observed before the treatment. These results show that cold-water stress increases phosphorylation of all tau species in mouse brain.

Phosphorylation of Tau in Brains of AD Model Mice Analyzed by Phos-Tag SDS-PAGE

We next examined the phosphorylation states of human P301L tau expressed in brains of JNPL3 transgenic mice, in which aggregates of the mutant tau are detected in animals >6.5 months of age (approximately 26W).^{26,47} We assessed mice at 24W and 49W. Sarkosyl-insoluble aggregated tau was found in JNPL3 mice at 49W but not at 24W (Figure 3A). Although sarkosyl-insoluble tau migrated as a single band slightly slower than sarkosyl-soluble tau in Laemmli SDS-PAGE (Figure 3A), it displayed smeared bands in the upper region of the Phos-tag SDS-PAGE gel (Figure 3A), confirming hyperphosphorylation of sarkosyl-insoluble tau. The sarkosyl-insoluble fraction contained a small amount of tau with normal phosphorylation states (Figure 3A), which might represent soluble tau trapped in aggregates. In contrast, soluble tau showed a banding pattern similar to that at 24W before aggregate formation (Figure 3A). These results indicate that sarkosyl-soluble P301L tau is not hyperphosphorylated in the brain even when tau aggregates are formed.

We also examined the phosphorylation states of endogenous tau in another mouse AD model, 5 × FAD, which has transgenes of human amyloid precursor protein and presenilin-1 with five familial AD mutations, leading to increased amyloid β peptide production.²⁸ Increased amyloid β peptide production is reported to induce tau hyperphosphorylation.^{9,48} Although an increase in tau phosphorylation is not detected in this mutant mouse when probed with AT8,²⁸ it may be detected if the total tau phosphorylation status is analyzed by Phos-tag SDS-PAGE. Therefore, we examined its phosphorylation at the ages of 12W

Figure 3 Phosphorylation of tau in brains from two mouse models of Alzheimer disease examined by Phos-tag SDS-PAGE. **A:** Phosphorylation of human tau in brains of JNPL3 mice at 24W and 49W. Tau was separated into sarkosyl-insoluble and -soluble fractions and detected by immunoblotting with Tau5 after Laemmli (**upper panel**) or Phos-tag (**lower panel**) SDS-PAGE. **B:** Phosphorylation of endogenous tau in brains of 5 × FAD mice at 12W and 48W. Tau was detected by immunoblotting with Tau5 after Laemmli (**upper panel**) or Phos-tag (**lower panel**) SDS-PAGE. Lae, Laemmli SDS-PAGE; P, sarkosyl-insoluble; Phos, Phos-tag SDS-PAGE; S, sarkosyl-soluble; WT, wild-type mice; 12W, 12 weeks of age; 24W, 24 weeks of age; 48W, 48 weeks of age; 49W, 49 weeks of age.

Figure 4 Phosphorylation states of tau in an elderly normal human brain. **A:** Phosphorylation states of tau in the extract of the brain of an elderly NC (case e) were analyzed by immunoblotting with HT7, RD3, and anti-4R after Laemmli (upper panels) or Phos-tag (lower panels) SDS-PAGE. The six rhTs are shown in the left lane. Densitometric scans of the NC human brain in the Phos-tag SDS-PAGE gels are shown at the right. Dotted vertical lines indicate the positions of the rhTs. The region scanned is indicated at the right side of the blots. **B:** Ser404 phosphorylation of tau in human brain. Tau in the brain extract of the NC (case e) was immunoblotted with HT7 and P-S404 after Phos-tag SDS-PAGE; the densitometric scans are shown at the right. Black indicates HT7; gray, P-S404. a.u., arbitrary unit; Lae, Laemmli SDS-PAGE; NC, normal control; Phos, Phos-tag SDS-PAGE; P-S404, phospho-Ser404; RD3, anti-3R tau; rhT, recombinant isoform of human tau.

and 48W, when amyloid plaques and neuron loss, respectively, are reported in the mutant mice.²⁸ Tau at both ages migrated almost identically to tau of wild-type mice (Figure 3B), indicating that hyperphosphorylated tau is scarcely present in $5 \times$ FAD mice.

Phosphorylation States of Tau in Human Brains

Before examination of tau in tauopathy brains, we compared the banding pattern of tau in a normal human brain at 77 years of age (Table 1) with the six recombinant human tau isoforms (2N4R, 2N3R, 1N4R, 1N3R, 0N4R, 0N3R). In Laemmli SDS-PAGE, normal human tau showed several bands within the region where recombinant tau migrated (Figure 4A). Human adult brain expresses both 3R and 4R tau.^{4,6,12,49–51} Immunoblotting with 3R- or 4R-specific antibodies revealed expression of the 0N and 1N isoforms of both 3R and 4R tau, whereas the 2N isoforms were less abundant (Figure 4A), consistent with previous results.^{14,15} To avoid weak reactivity of RD4 with human

4R tau owing to deamidation,²⁹ we used anti-4R tau to detect human 4R tau.²⁴

The six isoforms of recombinant tau were also separated according to their sizes in Phos-tag SDS-PAGE (Figure 4A and Supplemental Figure S1), as in Laemmli SDS-PAGE (Figure 4A), suggesting that nonphosphorylated tau shows the same mobility as the corresponding isoform of recombinant tau. Human tau appeared as at least eight distinct bands with smear staining in the upper region when probed with anti-human tau HT7 (Figure 4A). The Phos-tag blot of normal human brain showed immunoreactivity in the region above recombinant tau, whereas the Laemmli blot did not. Four of the more rapidly migrating bands in Phos-tag SDS-PAGE showed mobilities similar to the recombinant 0N and 1N isoforms of both 3R and 4R tau, although brain tau migrated slightly faster than the corresponding recombinant tau bands. To see if some of these bands contain proteolytic fragments of tau isoforms with a larger molecular size, we performed immunoblotting with the Tau-C antibody, whose epitope is in the extreme C-terminal region of tau.²³ Tau-C showed the similar banding pattern to that of HT7 within the molecular weight range that encompassed the main four tau bands (Supplemental Figure S2). The results suggest that the overlap by truncated tau is, if present, small. Several bands were found in the low molecular weight region, which were recognized by HT7 but not Tau-C. These might be tau fragments without the C-terminal region.

To distinguish 3R and 4R tau, we blotted them with RD3 and anti-4R tau (Figure 4A). 3R tau showed a banding pattern similar to total tau as detected with HT7. These blots suggested that the second and third bands from the bottom might correspond to 0N4R/1N3R and 1N4R, respectively. To confirm that the upper bands were phosphorylated, immunoblotting with anti-P-S404 was performed (Figure 4B). As expected, the P-S404 immunoreaction was observed only with the bands at the upper region of the blot compared with HT7 immunoblotting. Interestingly, a densitometric profile of the P-S404 reaction almost overlapped with that of HT7 in the upper region; P-S404 accounted for approximately 57% of total tau (Figure 4B), which agrees well with a mass spectrometry analysis of adult rat brains showing that 56% of tau was phosphorylated at Ser404.⁵²

Phosphorylation of Tau in Brains of Human Tauopathy Patients

To gain insight into the pathologic changes in tau, tau in brains of AD and CBD patients was examined by Laemmli and Phos-tag SDS-PAGEs. Immunoblotting with HT7 and AT8 after Laemmli SDS-PAGE is shown in Supplemental Figure S3. AT8 consistently detected the upper tau band [s] in the temporal lobe of AD brains at Braak stage VI. Immunoblotting with HT7 after Phos-tag SDS-PAGE is shown in Figure 5A. All six normal controls showed similar banding patterns (Figure 5A), suggesting that the above results on case e (Figure 4) are representative of the human

Figure 5 Phosphorylation of tau in brains of human individuals, including NCs and AD and CBD patients. **A:** Phosphorylation states of tau in human brains. The extracts of the TL (**upper panel**) and Pg (**lower panel**) prepared from NCs (a–f), AD V patients (g–i) and AD VI patients (j–l), and CBD patients (m–o) were analyzed by immunoblotting with HT7 after Phos-tag SDS-PAGE. **B:** Comparison of tau phosphorylation between the temporal lobe and precentral gyrus of a NC individual (case e) after Laemmli (**upper panel**) and Phos-tag (**lower panel**) SDS-PAGE. The six rhTs are shown in the **left lane**. Densitometric scans of the TL and Pg are shown at the right. **C:** Comparison of tau phosphorylation in human individuals, NC (case e), AD V (case h) and AD VI (case l), and in the TL and Pg of CBD (case o) by immunoblotting with HT7, RD3, and anti-4R tau after Phos-tag SDS-PAGE. **D:** Densitometric scans of tau in NC and AD V blotted with HT7, RD3, and anti-4R. **Arrowheads** in HT7 indicate nonphospho-tau, which decreases in AD V, and **arrowheads** in RD3 and anti-4R indicate tau bands that decrease in AD V; **arrows** indicate tau bands increased in AD V. AD, Alzheimer disease; a.u., arbitrary unit; CBD, corticobasal degeneration; Lae, Laemmli SDS-PAGE; NC, normal control; Pg, precentral gyrus; Phos, Phos-tag SDS-PAGE; RD3, anti-3R tau; rhT, recombinant isoform of human tau; TL, temporal lobe; V, Braak stage V; VI, Braak stage VI.

brain. In addition to the temporal lobe, which displays primary pathology in AD, the precentral gyrus was examined because that region is affected in CBD.⁵³ The banding patterns of tau in the normal controls appeared similar between the temporal lobe and the precentral gyrus, but to compare them more quantitatively, the sample of case e was run on the same blot and subjected to densitometry (**Figure 5B**). Tau in the temporal lobe was phosphorylated slightly more than that in the precentral gyrus (**Figure 5B**). AD patients at Braak stage V had tau with a little slower migration than the normal control in the temporal lobe (**Figure 5A**), and tau from patients at Braak stage VI displayed smeared staining in the upper region in both the temporal lobe and precentral gyrus (**Figure 5A**). CBD patients had a fraction of tau with a slightly slower mobility than tau of normal controls in the temporal lobe, and the amount increased in the precentral gyrus (**Figure 5A**).

Examples of the normal control (case e) and of AD at Braak stage V (case h) and VI (case l), and the temporal lobe and precentral gyrus of CBD (case o) were compared in more detail next to each other with the use of HT7 total tau antibody (**Figure 5C**). Tau from the AD brain at Braak stage V and the normal control was analyzed by densitometric scanning (**Figure 5D**). AD tau at Braak stage V was shifted upward slightly compared with that in the normal control.

The relative ratio of the nonphosphorylated forms of 0N3R, 1N3R/0N4R, and 1N4R tau decreased (**Figure 5D**) and that of upper phosphorylated bands increased in Braak V AD brain (**Figure 5D**). This tendency was more remarkable in an AD patient at Braak stage VI, which showed extremely shifted tau (**Figure 5C**). In a CBD patient, tau had a banding pattern similar to the AD tau at Braak stage V, and tau species in the precentral gyrus were slightly more phosphorylated than those in the temporal lobe.

Although both 3R and 4R tau isoforms accumulate as neurofibrillary tangles in AD, 4R tau preferentially aggregates in CBD.^{15,54–56} We asked whether 3R and 4R tau isoforms were similarly phosphorylated in AD or CBD brains with the use of normal human tau for reference (**Figure 5, C and D**). The 3R, rather than 4R, tau blot resembled that of total tau (HT7). The signal intensity of AD tau at Braak stage V increased in the upper bands for both 3R and 4R tau (**Figure 5D**) and decreased in the lower bands, corresponding to the main bands of the normal control (**Figure 5D**). Highly phosphorylated smeared tau was detected in both 3R and 4R tau at Braak stage VI. Comparison between 3R and 4R tau in the CBD patient showed that higher phosphorylated tau was detected in a blot of anti-4R. In addition, 4R tau isoforms were phosphorylated in precentral gyrus more than in the temporal

Figure 6 Phosphorylation states of sarkosyl-soluble and -insoluble tau in human tauopathy patients. **A:** Phos-tag SDS-PAGE immunoblot with HT7 of tau in S and P fractions of human brains. NC, case e; AD V, case h; AD VI, case l; CBD, case o. **B:** Phosphorylation states of S tau in the TL and Pg of brains from NC (a–f), AD V (g–i) and VI (j–l), and CBD (m–o) individuals as immunoblotted with HT7 after Phos-tag SDS-PAGE. AD, Alzheimer disease; NC, normal control; P, sarkosyl-insoluble; Pg, precentral gyrus; S, sarkosyl-soluble; TL, temporal lobe; V, Braak stage V; VI, Braak stage VI.

lobe, whereas phosphorylation states of 3R tau in CBD in the temporal lobe and precentral gyrus were similar, consistent with the histologic results (Supplemental Figure S3).

Phosphorylation of Soluble Tau in Brains of Human Tauopathy Patients

It is not yet known whether hyperphosphorylation of tau begins before or after aggregate formation.² We used the Phos-tag method to detect, if present, the intermediate phosphorylation states of tau in the soluble fraction. Sarkosyl-soluble tau was separated from insoluble tau by centrifugation.⁵⁷ The sarkosyl pellets were suspended in the same volume of SDS sample buffer as the supernatant fluids. Therefore, the immunoblot signals of the pellets represent the amounts of aggregated tau in three individuals. Insoluble tau, which was detected as smeared bands in the upper region of the gel, was increased markedly in AD at Braak stage VI (Figure 6A). Insoluble tau from a CBD patient was present in an amount intermediate between Braak stage V and VI of AD patients. The banding pattern of smeared tau appeared different between AD and CBD.

Soluble tau from normal controls and AD and CBD patients was compared in Figure 6B. Although there were slight differences in the banding patterns, tau from all individuals migrated similarly in Phos-tag SDS-PAGE. The position of the slowest migrating bands, which may represent the most highly phosphorylated species of tau in the soluble fraction, was similar among control and disease brains. Rather, the upper bands of soluble tau in AD at Braak stage VI (cases j, k, and l) were decreased compared with the control individuals (Figure 6B), suggesting that slightly higher phosphorylated tau in AD brains at Braak stage V (Figure 5) was already incorporated into aggregates. All of these results suggest that

phosphorylation of soluble tau is not increased in AD or CBD brains.

Comparison of Highly Phosphorylated Tau in Perinatal Mouse Brains and Brains of Cold Water–Treated Mice with AD Tau

Tau is highly phosphorylated in fetal to perinatal brains (Figure 1C), brains of mice treated with cold-water stress (Figure 2), and AD brains (Figure 5). However, phosphorylation levels across these three groups were not compared directly. We thus compared the electrophoretic mobilities of tau from these three sources in Phos-tag SDS-PAGE (Figure 7). Interestingly, perinatal tau and cold water–stressed tau showed similar mobilities. A main band in the brain of the cold water–treated mouse migrated to a position similar to one of two main bands in the perinatal brain (Figure 7), suggesting that these tau proteins had similar extents of phosphorylation. In contrast, AD tau showed a wide range of smeared staining with several recognizable bands in a region similar to visible tau bands in the cold water–stressed brains (Figure 7). These results suggest that tau phosphorylation levels are determined by a similar mechanism in perinatal and cold water–stressed mouse brains, which thus may share some common features with AD brains.

Discussion

Tau is phosphorylated in normal brains and hyperphosphorylated in AD brains.^{36,58} Although many phosphorylation sites were determined, it is not clear to what extent those sites are phosphorylated under physiologic or pathologic

Figure 7 Comparison of phosphorylation states of tau in mouse brain at P2, in the brain from a mouse treated with cold water, and in the brain of an individual with AD VI as detected with Tau5 antibody after Phos-tag SDS-PAGE. **White arrow** in the lane of cold water indicates the band with similar migration to a band of neonatal tau. **Arrows** in the lane of AD VI indicate tau bands migrating within the region of cold water–induced hyperphosphorylated tau. The **left lane** shows r-mtau 0N3R, 0N4R, and 1N4R. AD, Alzheimer disease; P2, postnatal day 2; r-mtau, recombinant mouse tau isoform; VI, Braak stage VI.

conditions. In particular, how much tau is nonphosphorylated in brains was not examined. In this study, we applied Phos-tag SDS-PAGE to the analysis of tau phosphorylation in mouse and human brains. The Phos-tag method enabled us to observe the total phosphorylation profiles of tau and revealed that substantial amounts of tau were nonphosphorylated in brains and that sarkosyl-soluble tau was not hyperphosphorylated in the brains from AD and CBD patients. These results indicate that tau exists in different phosphorylation states, including totally nonphosphorylated tau, in brains and suggest that hyperphosphorylation proceeds in aggregates.

The upward shift of electrophoretic mobility of tau in Laemmli SDS-PAGE is considered as an indication of phosphorylation, but its use is limited. We have shown here that phosphorylated tau was extremely shifted upward in Phos-tag SDS-PAGE, and the method is useful to observe overall phosphorylation of tau and for quantification of nonphosphorylated tau. It is well known that tau is highly phosphorylated in fetal/perinatal brains^{35,37,38} and in mice exposed to cold-water stress.^{32,33,46} Phos-tag SDS-PAGE not only successfully recapitulated elevated levels of phosphorylation but also systematically demonstrated the entire phosphorylation profile of tau in these contexts. Phos-tag SDS-PAGE also allowed for the first time a comparison of phosphorylation profiles in various contexts in a quantitative manner and suggested a similar mechanism underlying hyperphosphorylation of tau in brains stressed by cold-water treatment and in the perinatal brain (Figure 7). Considering that cold-water treatment preferentially suppresses the activity of protein phosphatases,⁴⁶ higher phosphorylation of perinatal tau may also be caused by reduced phosphatase activity, although high kinase activity was suggested.^{38,42} This is supported by the results of *in vitro* phosphorylation/dephosphorylation experiments (Figure 1, D and E); adult tau, which has a low level of phosphorylation, was phosphorylated in the presence of a phosphatase inhibitor more than perinatal tau, whereas perinatal tau, which has a high endogenous level of phosphorylation, displayed a lower extent of further phosphorylation than adult tau under the same conditions. These data support the idea that the phosphorylation states of tau in mouse brains could be determined primarily by the activity of protein phosphatase(s).^{45,59}

Although many sites are phosphorylated in tau *in vivo*, it is not known whether these sites are phosphorylated in a single tau molecule or in different tau molecules.⁶⁰ Tau was separated into more than four bands in adult mouse brains and more than eight bands in elderly human brains. Considering that proteins are shifted upward depending on the number and sites of phosphorylation in Phos-tag SDS-PAGE,^{21,34} these data indicate clearly that there are many tau species with different phosphorylation states. This was also the case when tau was highly phosphorylated in perinatal brains or brains of cold water-stressed mice. AD brains displayed a smear distribution of highly phosphorylated tau in Phos-tag SDS-PAGE. A similar smear reaction was seen in Laemmli SDS-PAGE, but the principle behind the upward

shift of tau during electrophoresis is different. Although we could not determine whether these smear bands were a result of phosphorylation and/or aggregates, Phos-tag SDS-PAGE of AD tau followed by immunoblotting showed several discrete bands, suggesting that a particular combination of sites is phosphorylated in AD tau. It will be interesting and important to analyze in more detail which sites are phosphorylated when and where in both control and AD brains.

Phosphorylation, including tyrosine phosphorylation, shifts the proteins upward in Phos-tag SDS-PAGE^{21,34}; therefore, a nonphosphorylated component can be separated from the phosphorylated populations. This enabled us to identify nonphosphorylated tau and to measure its ratio in total tau, regardless of the identification of phosphorylation sites. We used bacterially expressed tau for the reference for nonphosphorylated tau. Recombinant human tau isoforms were separated in the order of molecular size in Phos-tag SDS-PAGE (Supplemental Figure S1). Therefore, tau with electrophoretic mobility similar to recombinant tau was considered to be nonphosphorylated. Moreover, because a single antibody was used to detect different phosphorylation states of tau, we can estimate the ratio of tau with different phosphorylation states on a single blot.

One of the interesting findings in this study was the substantial amount of nonphosphorylated tau in mouse and human brains. Detection of totally unphosphorylated tau was lacking in previous studies in which antibodies for phosphorylated tau were mostly used. In contrast, biochemical studies usually estimate the average number of phosphorylation sites, for example, as 1 to 3 phosphates per normal tau and 5 to 10 phosphates per AD tau.^{58,61,62} We measured the extent of nonphospho-tau to be approximately 31% in adult JPNL3 mouse brain expressing the single isoform of human tau. Adult mice endogenously express two isoforms, 0N4R and 1N4R,¹⁴ of which nonphosphorylated 0N4R tau constituted approximately 30.9% of total tau. The case of human brain is more complicated because all six isoforms are expressed.¹⁵ When 3R and 4R tau were measured separately, nonphospho-0N3R tau was 17.9% of total 3R tau, and nonphospho-0N4R tau was approximately 14.1% of total 4R tau in a normal elderly individual (case e). We cannot exclude the possibility of dephosphorylation in human brains during postmortem delay, but a similar estimate was obtained with mouse brain, (Figure 1) from which tau was prepared in cold buffer that contained phosphatase inhibitors immediately after decapitation. A potential problem is if the proteolytic fragments of tau isoforms with a higher molecular weight overlap nonphospho-tau with a smaller molecular size. Tau is targeted by many proteases, including caspase, calpain, and asparagine endopeptidase.^{63–67} Calpain and asparagine endopeptidase produce fragments at around 17 kDa, 25 kDa, and 35 kDa smaller than the smallest 0N3R tau (Supplemental Figure S2). Caspase cleaves tau at Asp421 to generate the C-terminal truncated fragment,⁶³ which could have the similar mobility to nonphosphorylated tau. However, this seems unlikely because

the anti-Tau-C blot gave the similar banding intensity to the HT7 antibody blot. Therefore, we think that substantial amounts of tau are indeed present in a totally non-phosphorylated status in human brain.

We sought to identify the intermediate phosphorylation states of tau, which may represent an early step of hyperphosphorylation. At least to our knowledge, such intermediate phosphorylation states of tau were not described clearly. In addition, it is not yet known whether hyperphosphorylation occurs first on soluble or aggregated tau.² Here, we detected the increase in a slightly higher phosphorylated tau in AD at Braak stage V than in the normal control. Such tau was removed from the soluble supernatant fluid of AD brain extracts by ultracentrifugation, suggesting that the slightly higher phosphorylated tau is already included in aggregates. Although we could not identify these phosphorylated forms in the sarkosyl-insoluble fraction because of larger amounts of aggregated tau, we could conversely compare the phosphorylation profiles of soluble tau in normal, AD, and CBD brains. We found that higher phosphorylation did not occur in the soluble tau in either AD or CBD brains (Figure 6), in agreement with a biochemical study,⁵⁸ in which the same conclusion was derived from detailed elution profiles of proteolytic fragments of soluble tau prepared from normal and AD brains in liquid chromatography. The same results were obtained with FTDP-17 mutant tau in the JNPL3 mouse brain; soluble P301L tau from older mice that contained aggregates in the insoluble fraction showed a phosphorylation pattern identical to tau in younger mice that did not exhibit aggregation (Figure 3A). These results suggest that hyperphosphorylation occurs after incorporation of tau into aggregates. Otherwise, once soluble tau is highly phosphorylated, it may be incorporated into the aggregates immediately in diseased brains. It will be important to identify such intermediate phosphorylation states of tau to understand the initial stage of AD pathogenesis and its development at early stages.

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Supplemental Data

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