Imbalanced Expression of Tau and Tubulin Induces Neuronal Dysfunction in C. elegans Models of Tauopathy

Tomohiro Miyasaka1*, Yuki Shinzaki1, Satomi Yoshimura1, Sawako Yoshina2, Eriko Kage-Nakadai2,3, Shohei Mitani2 and Yasuo Ihara1

1 Department of Neuropathology, Faculty of Life and Medical Sciences, Doshisha University, Kyoto, Japan, 2 Department of Physiology, School of Medicine, Tokyo Women’s Medical University, Tokyo, Japan, 3 Graduate School of Human Life Science, Osaka City University, Osaka, Japan

Open Access

Edited by: Naruhiko Sahara, National Institute of Radiological Sciences (NIRS), Japan
Reviewed by: Emmanuel Planel, Laval University, Canada; Kentaro Tanemura, Tohoku University, Japan
*Correspondence: Tomohiro Miyasaka tomiyas@mail.doshisha.ac.jp

Specialty section: This article was submitted to Neurodegeneration, a section of the journal Frontiers in Neuroscience
Received: 27 February 2018
Accepted: 31 May 2018
Published: 20 June 2018

Tauopathy is a type of dementia defined by the accumulation of filamentous tau inclusions in neural cells. Most types of dementia in the elderly, including Alzheimer’s disease, are tauopathies. Although it is believed that tau protein abnormalities and/or the loss of its functions results in neurodegeneration and dementia, the mechanism of tauopathy remains obscure. Loss of microtubules and/or tubulin is a known consequence of tau accumulating in neurons in Alzheimer’s disease. In other words, there is an excess level of tau relative to tubulin in tauopathy neurons. To test whether this imbalance of tau and tubulin expression results in the neurotoxicity of tau, we developed several transgenic C. elegans lines that express human tau at various levels in pan-neurons. These worms showed behavioral abnormalities in a tau expression-dependent manner. The knockdown of a tubulin-specific chaperon, or a subset of tubulin, led to enhanced tau toxicity even in low-expressing tau-transgenic worms that showed no abnormal behaviors. In addition, the suppression of tau expression in tubulin knockdown worms rescued neuronal dysfunction. Thus, not only the overexpression of tau but also a reduction in tubulin can trigger the neurotoxicity of tau. Tau expressed in worms was also highly phosphorylated and largely bound to tubulin dimers rather than microtubules. Relative amount of tubulin-unbound tau was increased in high-expressing tau-transgenic worms showing tau toxicity. We further demonstrated that tau aggregation was inhibited by co-incubation of purified tubulin in vitro, meaning sufficient amounts of tubulin can protect against the formation of tau inclusions. These results suggest that the expression ratio of tau to tubulin may be a determinant of the tauopathy cascade.

Keywords: tau, tauopathy, microtubule, tubulin, neurodegeneration, C. elegans, Alzheimer’s disease

INTRODUCTION

In certain types of neurodegenerative diseases, affected neurons or glial cells have filamentous tau inclusions called neurofibrillary tangles, neuropil threads, and glial fibrillary tangles (Serrano-Pozo et al., 2011; Spillantini and Goedert, 2013). These disorders, which constitute the majority of age-dependent dementia, are called tauopathies and include Alzheimer’s disease (AD), frontotemporal dementia (FTD), and Parkinson’s disease (PD). Tauopathies are characterized by the accumulation of filamentous tau inclusions in neuronal cells.
imbalanced expression of tau and tubulin can induce tauopathy, tubulin triggers the tauopathy cascade. To confirm that the cells determines tau toxicity and that excess tau relative to tauopathy.

Not a passive event and is involved in the early pathogenesis of regardless of neurofibrillary tangle formation (Cash et al., 2003).

Furthermore, the pathogenic mutations in the tau gene have been identified from genetic studies of familial tauopathy, FTDP-17 (Iqbal et al., 2016). These findings indicate that changes in tau and its function lead to neurodegeneration and dementia.

Numerous studies revealed that pathological tau inclusions are composed of insoluble filaments, paired helical filaments (PHF) and straight filaments (Grundke-Iqbal et al., 1986; Greenberg and Davies, 1990; Lee et al., 1991; Fitzpatrick et al., 2017). This pathologically deposited tau is abnormally phosphorylated to a greater extent than physiological tau (Khatoon et al., 1992; Hanger et al., 2009). Since this abnormal phosphorylation disrupts the ability of tau to promote microtubule (MT) assembly (Yoshida and Ihara, 1993), it has been considered a key element of the pathology that underlies tauopathies. However, the pathological significance of the phosphorylation on individual sites of tau remains unclear. Other abnormal modifications, such as ubiquitination, acetylation, glycation, isomerization, and truncation, do not occur as often in pathological tau but were also identified (Miyasaka et al., 2005b; Iqbal et al., 2016). In contrast to the huge filamentous inclusions, it is currently assumed that the small soluble aggregates called oligomers are an essential part of the tau toxicity or substance that propagates tau pathology (Goedert et al., 2017; Shafiei et al., 2017). However, it is also suggested that unaggregated tau can affect neuronal function (Miyasaka et al., 2016; Xie and Miyasaka, 2016). Thus, the definitive trigger of tau, as well as which type of tau is toxic, is currently unknown.

Loss of MTs and/or tubulin is an invariable feature of tau-accumulating neurons in AD (Terry et al., 1964; Cash et al., 2003; Zhang et al., 2015). This inverse relationship is reproduced in the affected neurons of tauopathy animal models (Tatebayashi et al., 2002; Miyasaka et al., 2005a, 2016). Because the physiological function of tau is known to promote the assembly of tubulin or to stabilize MTs by binding to them, it is believed that the hyper-phosphorylation of tau may lead to MT loss (Bodea et al., 2016; Wang and Mandelkow, 2016). However, the finding that tau-knockout mice did not show obvious developmental abnormalities or brain function abnormalities, suggests that MT loss cannot simply be explained by a lack of tau (Harada et al., 1994; Kimura et al., 2014). Recently, we showed that the hyper-phosphorylation of tau in hypothermic brains did not directly detach tau from MTs and that forced MT destruction induced simultaneous tau liberation and phosphorylation in cultured cells (Planel et al., 2008; Miyasaka et al., 2010). Furthermore, a reduction of MTs was found in the neurons of AD brains regardless of neurofibrillary tangle formation (Cash et al., 2003). These findings suggest that MT loss (or tubulin reduction) is not a passive event and is involved in the early pathogenesis of tauopathy.

We speculated that the amount of the tubulin in neural cells determines tau toxicity and that excess tau relative to tubulin triggers the tauopathy cascade. To confirm that the imbalanced expression of tau and tubulin can induce tauopathy, we developed several lines of transgenic C. elegans that express various levels of human tau pan-neuronally, and then, we assessed the effects of tubulin knockdown.

# MATERIALS AND METHODS

## Development and Maintenance of Worm Strain

Development and maintenance of integrant worm lines were performed as previously described (Xie et al., 2014; Miyasaka et al., 2016). Briefly, human tau or DsRed cDNA was subcloned into the site downstream of “pan-neuronal” unc-119 promoter (pUnc119) of pFXneo-punc119 vector (Maduro and Pilgrim, 1995). The transgenes were injected into N2 together with a marker, pFXneo-Pges-1::EGFP. Germline transformation and generation of extrachromosomal arrays in C. elegans strains were performed according to the standard protocol (Mitani, 1995). Stable tau-transgenic (Tg)-lines, generated by UV irradiation, were backcrossed to N2 five times before analysis. Tg-strains used here were as follows: Mock-Tg (tmIs388), WT4R(L)-Tg (tmIs389), WT4R(H)-Tg (tmIs390), WT4R(ExH1)-Tg (tmIs763), and WT4R(ExH2)-Tg (tmIs765). For RNAi, tau-Tg worms were crossbred with rrf-3 mutant lines (Mock-Tg/rrf-3 and WT4R(L)/rrf-3). For mec-7 or mec-12 knockout, mec-7 (e1506) or mec-12 (tm5083) mutant lines were used. All C. elegans strains were maintained on a nematode growth medium (NGM) plate spread with E. coli OP50 under standard conditions (Miyasaka et al., 2016).

## Feeding RNAi

The RNA interference of C. elegans by feeding with dsRNA-expressing E. coli. was performed as previously described, with minor modifications (Kage-Nakadai et al., 2016). Complementary DNA fragments encoding human tau (748–1326 bp; corresponding to 2N4R isoform tau cDNA), K07H8.1 (619–1058 bp), and mec-12 (619–1058 bp) were subcloned into the multiple cloning site of the L4440 vector and transformed to the HT115 (DE3) E. coli. strain. The desired double-stranded RNA (dsRNA) was produced by bi-directional expression induced by 1 mM IPTG. The bacteria expressing dsRNA were spread on NGM media supplemented with ampicillin and IPTG. Synchronously cultured worms expressing dsRNA were spread on NGM media supplemented with ampicillin and IPTG. Synchronously cultured worms were grown on these RNAi plates for 4 days and subjected to behavioral and biochemical analyses. To ensure efficient knockdown in neuronal cells, the worm lines crossbred with rrf-3 were used for all RNAi experiments (Simmer et al., 2002).

## Behavioral Analyses

A touch assay was performed as previously described (Miyasaka et al., 2005a). Briefly, on the forth day after hatching the worms were isolated onto new 3.5-cm plates, and their escape reactions were assessed in response to gentle touch with an eyelash under a stereoscopic microscope. The number of responses to 10 touch trials, five for the anterior plus five for the posterior, were counted. Uncoordinated movements (Unc) were also assessed.
under a microscope. The severity of Unc was scored as follows: normal, wide bending with fast movement (2 point); Unc, slow movement (1 point); severe Unc, negligible movement (0 point). Twenty worms were used in each assay for each experiment, and the experiment was performed three to four times. Thus, 60–80 worms were assessed for each line. For the abnormality in *tbce-1* knockdown, the severities of Unc of all worms grown on knockdown plates were scored as follows: normal, wide bending with fast movement (2 point); Unc, slow movement (1 point); severe Unc, negligible movement and burst (0 point).

**Biochemical Analyses**

For biochemical analysis, worms were grown on NGM plates at large rectangular plates (No. 2 square schale, Eiken chemical co Ltd., Tokyo, Japan). Synchronized worms were harvested in M9 buffer and pelleted by brief centrifugation. After washing twice with M9 buffer (22 mM KH$_2$PO$_4$, 42 mM Na$_2$HPO$_4$, 85 mM NaCl, and 1 mM MgSO$_4$), the worm pellets were weighed and stored at −80°C. Total worm lysates were prepared as previously described (Miyasaka et al., 2016). The worm pellets were sonicated in sodium dodecyl sulfate (SDS) sample buffer (80-mM Tris-HCl, 2% SDS, 10% glycerol, 1% 2-mercaptoethanol, pH 6.8) and cleared by ultracentrifugation at 150,000 × g for 10 min at 20°C. The protein concentration for each sample was verified by Coomassie Brilliant Blue (Wako Pure Chemical Industries, Ltd., Osaka, Japan) staining on SDS-poly acrylamide gel electrophoresis (PAGE). Soluble fractions were subjected to SDS-PAGE followed by Western blotting. The MT-binding of expressed tau was analyzed as previously described (Miyasaka et al., 2016). Freshly harvested worms were homogenized in MS buffer (0.1 M MES, 1 mM EGTA, 1 mM MgSO$_4$, 2 mM DTT, and 0.5% Triton X-100, pH 6.8) containing 20 µM Taxol, 2 mM guanosine triphosphate (GTP), and protease inhibitors (Complete®, Sigma-Aldrich, St. Louis, MO, United States) and phosphatase inhibitors (1 mM Na$_3$VO$_4$, 1 mM NaF, 1 mM okadaic acid, and 1 mM b-glycerophosphate) at room temperature and then immediately chilled. After brief centrifugation, soluble fractions were centrifuged at 100,000 × g for 15 min at 2°C to obtain the soluble free tubulin and insoluble MT fractions. MT-unbound tau in free tubulin fraction was immunoprecipitated with H-150 anti-tau antibody bound on ProteinG-conjugated Dynabeads (Thermo Fisher Scientific Inc., Waltham, MA, United States) staining on SDS-poly acrylamide gel electrophoresis (PAGE). Soluble fractions were subjected to SDS-PAGE followed by Western blotting. The MT-binding of expressed tau was analyzed as previously described (Miyasaka et al., 2016). Four-day-old WT4R-Tg worm pellets were homogenized in ×10 volume of Tris-saline buffer (TS; 50 mM Tris, 150 mM NaCl, pH 7.6) containing protease and phosphatase inhibitors described above. After ultra-centrifugation at 120,000 × g for 15 min at 2°C, the soluble fractions were adjusted in 0.5 M NaCl and 2% 2-mercaptoethanol and heated at 100°C for 5 min. After the precipitates were removed by brief centrifugation, the soluble (heat-stable) fractions were precipitated by 50% ammonium sulfate. After

**Purification and Dephosphorylation of Tau Expressed in Worms and MT-Binding Assay**

Purification and dephosphorylation of tau and an MT-binding assay were performed as previously described with minor modifications (Xie et al., 2014). Four-day-old WT4R-Tg worm pellets were homogenized in ×10 volume of Tris-saline buffer (TS; 50 mM Tris, 150 mM NaCl, pH 7.6) containing protease and phosphatase inhibitors described above. After
centrifugation at 20,000 × g for 15 min at 2°C, the resultant pellets were re-solubilized and incubated with or without Lambda protein phosphatase (New England BioLabs, Inc., Ipswich, MA, United States) for 30 min at 30°C. Deynorphorylated and non-dephosphorylated tau were purified by using both heat and ammonium sulfate precipitation as described above. For the preparation of worm MT, fresh Mock-Tg worms were homogenized in ×5 volume of MS buffer containing protease and phosphatase inhibitors and centrifuged at 120,000 × g for 15 min at 2°C. Then, the soluble fraction was incubated with 20 µM Taxol and 2 mM GTP for 30 min at 25°C. After the addition of 0.5 M NaCl to remove the endogenous MAPs, polymerized MTs were collected by ultra-centrifugation and re-suspended into MS buffer containing Taxol and GTP. After the re-binding of purified tau on MTs at 25°C for 20 min, MT-unbound and MT-bound fractions were separated by ultra-centrifugation.

Morphological Analyses

Immunocytochemical analyses were performed by using the freeze-cracking method (Miyasaka et al., 2005a). Cracked worms put on PDL (poly-d-lysine)-coated glass slides were immersed in pre-cooled MeOH followed by hydration and permeabilization with 0.1% Triton X-100 in TS. After the worms were soaked with 10% goat serum in TS, the specimens were incubated with 1% BSA in TS containing primary antibodies. Bound antibodies were visualized using Alexa-conjugated secondary antibodies (Molecular Probes, Inc., Eugene, OR), and observed under an LSM700 microscope (Carl Zeiss Inc., Jena, Germany).

Drug Treatment

Trimethylamine-N-oxide (TMAO) was dissolved in H2O and stored in −20°C. For treatment, the compound was diluted with M9 buffer and directly added onto NGM plate. Worms were synchronized and grown on drug-containing plates for 3 days and subjected to biochemical analyses.

In Vitro Tau Aggregation Assay

Purification of recombinant tau corresponding to the 0N4R isoform and in vitro tau aggregation assays, were performed as previously described (Xie et al., 2014, 2015). Briefly, the recombinant tau 0N4R isoform expressed in E. coli [BL21(DE3)] was solubilized in homogenization buffer (50 mM PIPES, 1 mM EGTA, 1 mM DTT, and pH 6.8) with protease inhibitors and charged onto a phosphocellulose column (P11, Whatman). Tau protein fraction eluted in 0.1–0.3 M NaCl was precipitated by ammonium sulfate precipitation. The resultant pellet was re-solubilized by homogenization buffer containing 0.5 M NaCl and 1% 2-mercaptoethanol and fractionated by heat stability. The heat-stable fraction was further purified using reverse-phase HPLC (Cosmosyl protein-R, Nacalai tesque, Kyoto, Japan).
Purified porcine tubulin was purchased from Cytoskeleton Inc. (Denver, CO, United States). Purity and protein amount were analyzed by SDS-PAGE followed by Coomassie brilliant blue staining. For in vitro aggregation, 60 mg/ml of heparin was mixed with 10 mM purified tau, 100 mM NaCl, 10 mM thioflavine-T (Th-T), 10 mM HEPES (pH 7.4), and with/without tubulin. The time-dependent changes in Th-T fluorescence were measured at 465–635 nm (excitation) and 535–625 nm (emission) for 3 days. After incubation, the mixtures were centrifuged at 100,000 × g for 15 min before or after 1% Sarkosyl treatment. The fractions were analyzed by SDS-PAGE followed by CBB staining and Western blotting.

**Antibodies and Compounds**

The antibodies used here were as follows: pool-2 (anti-pan-tau, a generous gift of Dr. H. Mori), HT7 (anti-pan-tau, Innogenetics), H-150 (anti-human-tau, Santa Cruz Biotechnology, Dallas, TX, United States), AT8 (anti-phospho-tau, Thermo Fisher Scientific Inc.), tau-1 (anti-dephospho-tau, Merck Millipore, Billerica, MA, United States), PHF-1 (anti-phospho-tau, a generous gift of Dr. P Davies), pS262 (anti-phospho-tau, Sigma-Aldrich, St. Louis, MO, United States), DM1A (anti-α-tubulin, Sigma-Aldrich), KMX-1 (anti-β-tubulin, Merck Millipore), anti-mec-12 (6-11B1, Sigma-Aldrich), 1A4 (anti-α-actin, Sigma), and 6C5 (anti-GAPDH, Abcam). Taxol and trimethylamine-N-oxide were purchased from Sigma-Aldrich. Other materials were purchased from Nachalai tesque unless otherwise specified (Kyoto, Japan).

**Statistical Analysis**

All statistical analyses were performed using IBM SPSS statistics 25 (IBM, Armonk, NY, United States). Analysis of variance (ANOVA) was used for comparisons made between three or more groups, followed by Tukey's post hoc test. All data were presented as the means ± SEM. In all cases, *P* values lower than 0.05 were considered as significant.

**RESULTS**

To date, a number of Tg mouse lines have been developed (Noble et al., 2010), with varying neurological phenotypes. Although some of the unique phenotypes may be caused by...
the promoter used for exogenous tau expression in particular lines, it is generally understood that the expression level of tau correlates with the severity of the pathology in Tg mice. To probe whether the amount of tau expression affects its neuronal phenotype, we developed several lines of tau-expressing worms using the pan-neuronal punc-119 promoter (Xie et al., 2014; Miyasaka et al., 2016). Integration of extrachromosomal arrays in the worm genome allows for the introduction of various numbers of transgene copies (Mitani, 1995). We isolated four independent integrant human wild-type ON4R-tau-Tg lines with different expression levels (Figure 1A). WT4R(H)-Tg, WT4R(ExH1)-Tg, and WT4R(ExH2)-Tg lines express tau at 2.17 ± 0.17, 2.98 ± 0.24, and 3.16 ± 0.31 fold than WT4R(H)-Tg respectively. We also found the amount of α-tubulin expression was slightly but significantly higher in WT4R(Ex2)-Tg line. As shown in Figure 1B, lines with extremely high tau expression levels, WT4R(ExH1)-Tg, expressed (Miyasaka et al., 2016). Furthermore, most of the times more tau had a more severe Unc phenotype as previously minimal Unc but the WT4R(H)-Tg worms expressing three Tg line. As shown in expression was slightly but significantly higher in WT4R(Ex2)-Tg respectively. We also found the amount of MTs and/or tubulin is inversely correlated with tau-accumulating neurons (Terry et al., 1964; Zhang et al., 2015). Therefore, we hypothesize that the amount of MTs may determine a threshold of neurotoxicity induced by tau. To test whether the expression level of tubulin affects tau toxicity, we sought to reduce tubulin neurotoxicity induced by tau. To test whether the expression level of tubulin affects tau toxicity, we sought to reduce tubulin expression in tau expressing worms. Because there are nine α-tubulin genes and six β-tubulin genes in C. elegans (Gogonea et al., 1999), a direct knockdown of a single tubulin isotype may not be sufficient to reduce the level of total tubulin in neurons. Therefore, we chose the tbce-1 gene, a homologue of human tubulin-folding cofactor E (tbce), a tubulin chaperon needed for de novo synthesis of α-tubulin (Tian and Cowan, 2013; Nithianantham et al., 2015). A missense mutation of the Tbce gene is identified in the spontaneous mouse model of progressive motor neuronopathy (Bommel et al., 2002; Martin et al., 2002), indicating that the functional decline of TBCE can induce MT abnormality in neurons. In C. elegans model, target genes can be knocked down easily by feeding E. coli expressing sense/antisense RNA in vivo. As shown in Figure 2, tbce-1 knockdown by feeding-RNAi resulted in the reduction of α-tubulin and induced abnormal Unc behavior. In severe cases, the worms showed complete immobility and burst (Figure 2A). The severity of unc was significantly increased in human-tau expression (Figure 2B). tbce-1 knockdown showed reduced expression of α-tubulin (Figures 2C,D). Intriguingly, tbce-1 knockdown increased tau expression (Figure 2D). Thus, the low expression of tau can affect neuronal functions when the expression of tubulin is reduced.

To address whether tubulin knockdown directly enhances tau-induced neuronal dysfunction, we reduced the expression of mec-12, an isotype of α-tubulin specifically expressed in touch neurons (Fukushige et al., 1999), in WT4R(L)-Tg/rrf-3 worms, which express human-tau (Figure 3A). This is probably the only in vivo experimental model in which the relationship between specific tubulin isoform and neural function is strictly corresponded. Knocking-down mec-12 in wild-type worms showed touch sense abnormality (Figures 3B,C), a phenotype of mec-12 mutant worms. When the mec-12 RNAi was applied to Mock-Tg and WT4R(L)-Tg worms in the rrf-3 background, we found that the touch sense abnormality was significantly exacerbated in the WT4R(L)-Tg line (Figures 3B,C).
Control RNAi (Figures 3B,C) on WT4R(L)-Tg/rrf-3 worms did not cause any tactile abnormalities. Thus, α-tubulin downregulation alone can cause abnormalities in touch neurons, which are enhanced by the expression of human-tau. We addressed whether these exacerbations were specific to tau and found that the toxicity of tubulin knockdowns or tubulin chaperon knockdowns was not enhanced by DsRed expression by the same Unc-119 promoter (data not shown). Furthermore, the knockdown of genes unrelated to tubulin or MTs, including gfp, far-3, mtl-1, and col-149, did not show any abnormalities in tau-Tg worms (data not shown).

These data indicate that the relative levels of tau and tubulin are important regulators of tau toxicity. We next tested whether the abnormalities of mec-12 RNAi worms can be rescued by reducing tau expression. Reduced expression of tau by simultaneous RNAi knockdown significantly rescued touch sense abnormalities induced by mec-12 RNAi in WT4R(L)-Tg/rrf-3 worms (Figure 4). Thus, excessive expression of tau against tubulin is critical for tau-induced neurotoxicity.

How does the imbalance of tau and tubulin (or MTs) cause neurotoxicity? We first analyzed the properties of the MT-binding of tau expressed in the transgenic worms. MT-bound tau and free MT-unbound tau were fractionated...
Miyasaka et al. Tubulin Expressions Determines Tau Pathogenesis

after stabilizing MTs by Taxol. We found that human-tau, even in WT4R(L)-Tg worms, was almost completely recovered in the MT-unbound fraction (Figure 5). Because tau in transgenic worms is phosphorylated to a higher extent similar to PHF-tau (Miyasaka et al., 2016), we suspected that this may be caused by the abnormal phosphorylation. To investigate this, we purified human-tau from tau-Tg worms and tested their MT-binding after dephosphorylation. As shown in Figure 6, dephosphorylation by lambda protein phosphatase induced a large electrophoretic mobility shift and resulted in the loss of immunoreactivity for phosphorylation-dependent antibodies. Interestingly, dephosphorylation also resulted in the increased binding of tau to MTs (Figure 6). This suggests that the MT itself or MT-binding of tau is not a plausible explanation for how the imbalance of tau and tubulin causes neurotoxicity.

Recently, it has been suggested that tau also interacts with tubulin dimer(s) (Li et al., 2015; Li and Rhoades, 2017). To test whether MT-unbound tau is associated with tubulin dimers, we immunoprecipitated tau from tau-Tg worms and performed Western blotting. As shown in Figure 7, both α- and β-tubulin were co-immunoprecipitated with soluble tau (Figure 7), despite tau being unbound to MTs (Figure 5), indicating that tau binds to tubulin dimers. Quantitative analyses showed that the higher amount of tau was recovered from WT4R(H)-Tg than WT4R(L)-Tg. However, the relative amount of co-purified α- and β-tubulins were significantly lower in WT4R(H)-Tg than that of tau. This finding indicates that free-tau, which does not bind to either MTs or tubulin, may increase when the expression levels of tau are high. Approximately equal proportion of α- and β-tubulins were recovered in tau-bound fraction, suggesting that the α/β-tubulin dimers are associated with soluble tau (Figure 7D). The binding between tubulin dimers and MT-unbound tau is also confirmed in the mouse brain (data not shown).

The abundance of free-tau in neurons is a possible cause of tau toxicity. If so, it is conceivable that enhanced binding of tau to MT or tubulin may rescue tau neurotoxicity. TMAO is a chemical osmolyte that can stabilize protein conformations and is known to restore the MT-binding ability of phosphorylated tau in vitro (Tseng et al., 1999; Smith et al., 2000). WT4R(H)-Tg worms were grown on media containing TMAO and subjected to behavioral and biochemical analyses. As shown in Figure 8, the number of Tg worms having the Unc phenotype was significantly reduced by TMAO in a dose-dependent manner (Figure 8A). TMAO did not affect the expression of tau. However, high-dose of TMAO slightly enhanced the expression of tubulin (Figures 8B,C). Thus, the optimization of protein conformations...
and/or protein-protein interactions can ameliorate the neuronal dysfunction of WT4R(H)-Tg worms.

We further addressed whether tubulin directly attenuates tau aggregation, a typical abnormality identified in tauopathy neurons. As shown in Figure 9, recombinant tau formed Th-T-positive insoluble aggregates following co-incubation with heparin (Figure 9A). When similar amounts of purified tubulin and tau were added to the mixture, the Th-T values immediately increased. Because MT formations can occur under these conditions, the transient increase in Th-T fluorescence may be caused by MT-formation (Ackmann et al., 2000), but not tau aggregation. However, after 24 h, the Th-T fluorescence decreased and by 48 h, and the fluorescence was significantly lower than control values (Figure 9A). After 72 h of incubation, Sarkosyl-insoluble tau, corresponding to the aggregated tau, was verified. Figure 9B indicates that the Sarkosyl-insoluble tau emerged only in the presence of heparin. Co-incubation of equivalent tubulin with tau reduced the amount of tau fractionated into the Sarkosyl-insoluble fraction (Figure 9B). Although the lower (one-fifth) amount of tubulin did not show a transient Th-T increase, a weak but significant Th-T reduction was observed at 72 h. Taken together with the TMAO results, these data indicate that enhancing the binding of tau to tubulins may help reduce tau pathology through the reduction of neuronal dysfunction and tau aggregation.

**DISCUSSION**

Here, we demonstrated that not only an overexpression of tau but also a reduction of tubulins enhances tau-related pathology in worm models of tauopathy. Neuronal function was consistently rescued by either the suppression of tau expression or the pharmacological stabilization of tau/tubulin-binding. Furthermore, tubulin inhibited tau aggregation promoted by

---

**FIGURE 7** | MT-unbound tau in tau-Tg worms interacts with α/β-tubulin dimers in the soluble fraction. MT-unbound soluble fractions were prepared from Mock-Tg (a), WT4R(L)-Tg (b), and WT4R(H)-Tg (c) worms and subjected to the immunoprecipitation using anti-tau IgG (H-150). (A) Expression of tau, α-tubulin, and α-actin in the total lysate (input) are shown. (B) Immunoprecipitated proteins were analyzed using anti-tau (HT7), anti-α-tubulin (DM1A), and anti-β-tubulin (KMX-1) antibodies. (C) Relative amount of tau and α/β-tubulin in the co-immunoprecipitated fraction from tau-Tg worms were quantified (mean ± SEM, n = 6). (D) Quantitative analysis of each protein indicates that the nearly equal amount of α- and β-tubulin were co-immunoprecipitated with tau. Std. indicates the purified recombinant tau (for HT7) or porcine tubulins (for DM1A and KMX-1). Statistical significance was analyzed by Tukey’s post hoc test (**p < 0.01).
heparin in vitro. These data indicate that an imbalanced expression of tau compared to tubulin, specifically an excess of tau, is detrimental.

A loss of MTs and tubulin have been reported in the neurons of AD brains (Terry et al., 1964; Cash et al., 2003; Zhang et al., 2015). Interestingly, MT loss was found in neurons without neurofibrillary tangle formations, suggesting that the MT loss occurs independent of or ahead of the tangle formation. It is well known that tubulins are vulnerable proteins and need energy or co-factors to function properly (Arai et al., 1975). Thus, it is reasonable that the chronic decrease in metabolism, or the increased impairment of the MT protection and/or repair systems, will read the fray of cytoskeleton in the long-lived neurons.

Age is considered as a strong risk factor in neurodegenerative diseases such as AD. Various age-related abnormalities in cellular systems are considered in neurons (Baker and Petersen, 2018), and it is plausible that one or some of them are involved in triggering of tauopathy. Although further analysis is needed, dysregulation of the neuronal cytoskeletal proteins can also be defined as one character of brain protein aging, which may leads to the onset of neurodegenerative diseases. In the natural course of aging, the destruction of MTs has not been identified in nematodes or mice. Considering the lifetime of them, it is reasonable to assume that it takes longer time for this phenomenon to occur. In that sense, we cannot deny the possibility that the age-dependent tubulin/MT loss is a phenomenon unique to the neurons in the human brain that can live for decades.

In physiological condition, a much higher amount of tubulin compared to tau is present in brains. Our preliminary study indicated that there is roughly a 60-fold higher concentration of tubulin than tau in adult mice brains (data not shown). Although, it is difficult to estimate the local concentration of tau and tubulin in each cell type (ex. neuron, glia) or in each subcellular compartment (ex. cell body, axon, and dendrite), the above estimate indicates that slight changes of tau expression or tubulin reduction may not immediately tip their balance. This is consistent with the finding that it takes 8 months to develop pathology even in the PS19 line expressing P301S-tau at a five-fold higher level than endogenous tau (Yoshiyama et al., 2007). Interestingly, in the R406W-tau Tg mice, which have a lower level of tau and show mild pathology only after 18 months of age (Tatebayashi et al., 2002), tau aggregates were sparsely observed in neurons exhibiting tubulin-loss. Therefore, we speculate that tau pathology could result from not only aberrant expression of tau but also tubulin loss in aged neurons. It is also possible that once the aggregation of tau is initiated,
MT-degeneration is stimulated (Alonso et al., 1997). Thus, tau aggregation and tubulin degeneration may be developed cooperatively.

Here, we showed an interaction between tau and tubulin dimers in the MT-unbound fraction prepared from worms. This is reproduced in mouse brains (data not shown). Recently, it has been demonstrated that tau can bind to tubulin dimers and form multiple oligomers in vitro (Elbaum-Garfinkle et al., 2014; Li et al., 2015; Li and Rhoades, 2017). Because tau binds to the C-terminal region of tubulin at sub-micromolar Kd, it is reasonable that virtually all tau molecules are bound to MT or soluble tubulin dimers in healthy neurons (Fauquant et al., 2011; Lefevre et al., 2011). We could not distinguish by ultracentrifugation if the complex consisted of simple trimers of tau and α/β-tubulin dimers, or multiple complexes like small soluble MTs. However, it is likely that the MT-unbound tau does not behave alone despite its tau/MTs binding ability. Therefore, the regulatory mechanism for tau-MT binding by phosphorylation could be more complicated than has ever been considered (Bodea et al., 2016; Wang and Mandelkow, 2016).

Both of the responsible regions for MT/tubulin-binding and tau-tau binding (for aggregate formation) are located in the C-terminal region of tau containing MT-binding repeats (Gustke et al., 1994; Perez et al., 1996; Goode et al., 1997; Kadavath et al., 2015; Fitzpatrick et al., 2017; Lathuiliere et al., 2017). We also showed that the C-terminal fragment, including the MT-binding repeats, is responsible for the neurotoxicity of tau (Xie et al., 2014). Simply, it is conceivable that the interaction of an unknown factor(s), or tau-itself on a naked MT-binding domain, is crucial for exerting tau toxicity. Thus, as shown here, the binding of tubulin (or MT) on an MT-binding domain of tau, interferes with the undesirable interaction with other factors, including tau-itself. Because the affinity of tau-tau binding is about 20 times higher than that of the tau/tubulin interaction, a sufficient amount of tubulin may be necessary to suppress the neurotoxicity of tau (Lai et al., 2016). If the level of tubulins
declines due to aging or metabolic impairment, neurons may become prone to have higher levels of free tau and eventually tau aggregates.

In summary, our data suggested that the imbalanced expression of tau vs. tubulin might be a key step in the pathogenesis of tauopathy, and “free-tau” may be a toxic species of tauopathy. Thus, we propose the following hypothesis. Tau has the potential to be cytotoxic due to its MT-binding repeats. However, in healthy neurons MTs or tubulin dimers bind on the MT-binding repeats, thereby masking the toxicity. However, in some aged neurons in the elderly, a decline of tubulin and/or ectopic expression of tau occur by energy failure, oxidative stress, or amyloid depositions, which need to be experimentally identified. This will result in genuine “free-tau” that has free, exposed MT-binding repeats, which behave as either a toxic factor by itself, or promotes the self-aggregation of tau.

REFERENCES

Ackmann, M., Wiech, H., and Mandelkow, E. (2000). Nonsaturable binding indicates clustering of tau on the microtubule surface in a paired helical filament-like conformation. J. Biol. Chem. 275, 30335–30343. doi: 10.1074/jbc.M002590200

Alonso, A. D., Grundke-Iqbal, L., Barra, H. S., and Iqbal, K. (1997). Abnormal phosphorylation of tau and the mechanism of Alzheimer neurofibrillary degeneration: sequestration of microtubule-associated proteins 1 and 2 and the disassembly of microtubules by the abnormal tau. Proc. Natl. Acad. Sci. U.S.A. 94, 298–303. doi: 10.1073/pnas.94.12.298

Arai, T., Ibara, Y., Arai, K., and Kaziro, Y. (1975). Purification of tubulin from bovine brain and its interaction with guanine nucleotides. J. Biochem. 77, 647–658. doi: 10.1093/oxfordjournals.jbchem.a130767

Baker, D. J., and Petersen, R. C. (2018). Cellular senescence in brain aging and neurodegenerative diseases: evidence and perspectives. J. Clin. Invest. 128, 1208–1216. doi: 10.1172/JCI95145

Boede, L. G., Eckert, A., Ittner, L. M., Piguet, O., and Gotz, J. (2016). Tau physiology and pathomechanisms in frontotemporal lobar degeneration. J. Neurochem. 138(Suppl. 1), 71–94. doi: 10.1111/jn.13600

Bommel, H., Xie, G., Rossoll, W., Wiese, S., Jablonka, S., Boehm, T., et al. (2002). Missense mutation in the tubulin-specific chaperone E (Tbec) gene in the mouse mutant progressive motor neuronopathy, a model of human motoneuron disease. J. Cell Biol. 159, 563–569. doi: 10.1083/jcb.200208001

Cash, A. D., Aliev, G., Siedlak, S. L., Nunomura, A., Fujikoa, H., Zhu, X., et al. (2003). Microtubule reduction in Alzheimer’s disease and aging is independent of tau filament formation. Am. J. Pathol. 162, 1623–1627. doi: 10.1016/S0002-9440(10)62926-4

Delacourte, A., David, J. P., Sergeant, N., Buee, L., Wattez, A., Vermeersch, P., et al. (1999). The biochemical pathway of neurofibrillary degeneration in aging and Alzheimer’s disease. Neurology 52, 1158–1165. doi: 10.1212/WNL.52.6.1158

Elbaum-Garfinkle, S., Cobb, G., Compton, J. T., Li, X. H., and Rhoades, E. (2014). Fitzpatrick, A. W. P., Falcon, B., He, S., Murzin, A. G., Murshudov, G., Garringer, H. J., et al. (2017). Cryo-EM structures of tau filaments from Alzheimer’s disease. Nature 547, 185–190. doi: 10.1038/nature23002

Fukushige, T., Siddiqui, Z. K., Chou, M., Culotti, J. G., Gogonea, C. B., Siddiqui, S. S., et al. (1999). MEC-12, an alpha-tubulin required for touch sensitivity in C. elegans. J. Cell Sci. 112(1 Pt 3), 395–403.

AUTHOR CONTRIBUTIONS

TM and YI designed the experiments. TM, YS, and SatY performed the behavioral and biochemical analyses. SawY, EK-N, and SM developed the C. elegans models. TM wrote the manuscript.

FUNDING

This work was supported in part by the Grant-in-Aid for Scientific Research on Innovative Areas “Brain Protein Aging and Dementia Control” (TM 26117004), the “Integrated research on neuropsychiatric disorder”, which was carried out under the Strategic Research Program for Brain Sciences (TM and YI), and the JSPS KAKENHI Grant Numbers 20700324 (TM) and 22650074 (TM).
Shafiei, S. S., Guererro-Munoz, M. J., and Castillo-Carranza, D. L. (2017). Tau oligomers: cytotoxicity, propagation, and mitochondrial damage. *Front. Aging Neurosci.* 9:83. doi: 10.3389/fnagi.2017.00083

Simmer, F., Tijssen, M., Parrish, S., Koushika, S. P., Nonet, M. L., Fire, A., et al. (2002). Loss of the putative RNA-directed RNA polymerase RRF-3 makes *C. elegans* hypersensitive to RNAi. *Curr. Biol.* 12, 1317–1319. doi: 10.1016/S0960-9822(02)01041-2

Smith, M. J., Crowther, R. A., and Goedert, M. (2000). The natural osmotolyte trimethylamine N-oxide (TMAO) restores the ability of mutant tau to promote microtubule assembly. *FEBS Lett.* 484, 265–270. doi: 10.1016/S0014-5793(00)02169-4

Spillantini, M. G., and Goedert, M. (2013). Tau pathology and neurodegeneration. *Lancet Neurol.* 12, 609–622. doi: 10.1016/S1474-4422(13)70096-5

Tatebayashi, Y., Miyasaka, T., Chui, D. H., Akagi, T., Mishima, K., Iwakasi, K., et al. (2002). Tau filament formation and associative memory deficit in aged mice expressing mutant (R406W) human tau. *Proc. Natl. Acad. Sci. U.S.A.* 99, 13896–13901. doi: 10.1073/pnas.2022055999

Terry, R. D., Gonatas, N. K., and Weiss, M. (1964). Ultrastructural studies in Alzheimer's presenile dementia. *Am. J. Pathol.* 44, 269–297.

Tian, G., and Cowan, N. J. (2013). Tubulin-specific chaperones: components of a molecular machine that assembles the alpha/beta heterodimer. *Methods Cell Biol.* 115, 155–171. doi: 10.1016/B978-0-12-407577-0.00011-6

Tseng, H. C., Lu, Q., Henderson, E., and Graves, D. J. (1999). Phosphorylated tau can promote tubulin assembly. *Proc. Natl. Acad. Sci. U.S.A.* 96, 9503–9508. doi: 10.1073/pnas.96.17.9503

Wang, Y., and Mandelkow, E. (2016). Tau in physiology and pathology. *Nat. Rev. Neurosci.* 17, 5–21. doi: 10.1038/nrn.2015.131

Xie, C., and Miyasaka, T. (2016). The role of the carboxyl-terminal sequence of tau and MAP2 in the pathogenesis of dementia. *Front. Mol. Neurosci.* 9:158. doi: 10.3389/fnmol.2016.00158

Xie, C., Miyasaka, T., Yoshimura, S., Hatsuta, H., Yoshina, S., Kage-Nakadai, E., et al. (2014). The homologous carboxyl-terminal domains of microtubule-associated protein 2 and Tau induce neuronal dysfunction and have differential rates in the evolution of neurofibrillary tangles. *PLoS One* 9:e97996. doi: 10.1371/journal.pone.0097996

Xie, C., Sooda, Y., Shinzaki, Y., In, Y., Tomooko, I., Ibara, Y., et al. (2015). Identification of key amino acids responsible for the distinct aggregation properties of microtubule-associated protein 2 and tau. *J. Neurochem.* 135, 19–26. doi: 10.1111/jnc.13228

Yoshida, H., and Ibara, Y. (1993). Tau in paired helical filaments is functionally distinct from fetal tau: assembly incompetence of paired helical filament-tau. *J. Neurochem.* 61, 1183–1186. doi: 10.1111/j.1471-4159.1993.tb03422.x

Yoshimura, Y., Higuchi, M., Zhang, B., Huang, S. M., Iwata, N., Saito, T. C., et al. (2011). Synapse loss and microglial activation precede tangles in a P301S tauopathy mouse model. *Neuron* 53, 337–351. doi: 10.1016/j.neuron.2007.01.010

Zhang, F., Su, B., Wang, C., Siedlak, S. L., Mondragon-Rodriguez, S., Lee, H. G., et al. (2015). Posttranslational modifications of alpha-tubulin in Alzheimer disease. *Transl. Neurodegener.* 4:9. doi: 10.1186/s41035-015-0030-4

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Miyasaka, Shinzaki, Yoshimura, Yoshina, Kage-Nakadai, Mitani and Ibara. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.