Visualization of soluble tau oligomers in TauP301L-BiFC transgenic mice demonstrates the progression of tauopathy

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ABSTRACT

Accumulation of abnormal tau aggregates in the brain is a pathological hallmark of multiple neurodegenerative disorders including Alzheimer’s disease. Increasing evidence suggests that soluble tau aggregates play a key role in tau pathology as neurotoxic species causing neuronal cell death and act as prion-like seeds mediating tau propagation. Despite the pathological relevance, there is a paucity of methods to monitor tau oligomerization in the brain. As a tool to monitor tau self-assembly in the brain, we generated a novel tau transgenic mouse, named TauP301L-BiFC. By introducing bimolecular fluorescence complementation technique to human tau containing a P301L mutation, we were able to monitor and quantify tau self-assembly, represented by BiFC fluorescence in the brains of transgenic TauP301L-BiFC mice. TauP301L-BiFC mice showed soluble tau oligomerization from 3 months, showing significantly enriched BiFC fluorescence in the brain. Then, massive tau fragmentation occurred at 6 months showing dramatically decreased TauP301L-BiFC fluorescence. The fragmented tau species served as a seed for insoluble tau aggregation. In a result, insoluble TauP301L-BiFC aggregates coaggregated with endogenous mouse tau accumulated in the brain, showing subsequently increased BiFC fluorescence from 9 months. Neuronal degeneration and cognitive deficits were observed from 12 months of age. TauP301L-BiFC mouse model demonstrated that methylene blue reduced the amount of soluble tau oligomers in the brain, resulting in the prevention of cognitive impairments. We assure that TauP301L-BiFC mice are a bona-fide animal tool to monitor pathological tau oligomerization in AD and other tauopathies.

1. Introduction

Tau is a highly soluble, microtubule-associated protein that is abundantly expressed in neurons (Shin et al., 1991). In healthy neurons, tau stabilizes microtubules and promotes microtubule assembly (Cleveland et al., 1977; Kolarova et al., 2012; Mazanetz and Fischer, 2007; Obulesu et al., 2011). Under pathological conditions, tau dissociates from microtubules and becomes aggregated into insoluble filaments (Brunden et al., 2008; Iqbal et al., 2009; Lasagna-Reeves et al., 2011; Reddy, 2011). Accumulation of tau inclusions is a pathological characteristic of multiple neurodegenerative disorders collectively termed tauopathies, which include Alzheimer’s disease (AD) and frontotemporal dementia (FTD). Accordingly, several studies have focused on characterizing insoluble tau filaments known as neurofibrillary tangles (NFTs). However, accumulating evidence suggests that soluble tau oligomers, rather than insoluble filaments, are the neurotoxic species in the brain that lead to neuronal death and mediate tau propagation in the brain (Lasagna-Reeves et al., 2012a; Patterson et al., 2011a).

Tau oligomers are soluble forms of tau aggregates. Their sizes range from dimers to prefibrillar forms (Kaniyappan et al., 2017a). In studies of AD patients and tau animal models, tau oligomers were observed at early stages of pathogenic cascades, and the levels of tau oligomers correlated with memory deficits (Berger et al., 2007; Maeda et al., 2006, 2007). When extracellularly applied to cells, tau oligomers induced neuritic degeneration and neuronal loss, and increased reactive
oxygen species (Kaniyappan et al., 2017b; Usenovic et al., 2015). It is also reported that tau oligomers perturbed fast axonal transport of membranous organelles along microtubules (Swanson et al., 2017). In vivo studies demonstrated that intra-hippocampal injection of tau oligomers led to memory impairments in non-mutated tau expressed mice (Fa et al., 2016; Gerson et al., 2016; Koss et al., 2016; Puzzo et al., 2017). Alongside neurotoxicity, tau oligomers also play a critical role in disease spreading (Clavaguera et al., 2009; Frost et al., 2009). Swanson et al. (2017) and Fa et al. (2016) observed that tau oligomers were more actively internalized into cells compared to tau monomers or insoluble aggregates, and the internalized tau oligomers provoked intracellular tau aggregation (Gerson et al., 2016; Manassero et al., 2017; Swanson et al., 2017; Usenovic et al., 2015).

Despite the importance of tau oligomers, tools to investigate soluble tau oligomers in vivo are limited. Classically, tau oligomers were characterized from brain lysates by molecular weight differences on gel filtration chromatography (Ren and Sahara, 2013). Although this method provides quantitative information on tau oligomers, the status of tau oligomers may change during the sample preparation step, since tau oligomerization events are highly dynamic depending on the concentration and environment (Sahara et al., 2007). To overcome these limitations, Patterson et al. and Lasagna-Reeves et al. developed tau oligomer-specific antibodies, TOC1 (Patterson et al., 2011b) and T22 (Lasagna-Reeves et al., 2012b). These antibodies are useful for tau oligomer detection. However, the antibody specificity needs to be evaluated carefully, since tau oligomers are extremely heterogeneous species depending on their isoforms and post-translational modifications (Büe et al., 2000). Indeed, TOC1 and T22 are known to lose their selectivity to tau oligomers depending on experimental materials and conditions (Lasagna-Reeves et al., 2012b; Ward et al., 2013). To overcome the limitations of chromatography and loss of antibody selectivity, an easy and robust tool to monitor tau oligomerization in living systems is required. To this end, our group applied bimolecular fluorescence complementation (BiFC) technology to tau (Tak et al., 2013). In this system, full-length human tau protein is fused to non-fluorescent N- and C- terminal compartments of Venus fluorescent protein and expressed together in a cell. As a fluorescence turn-on sensor, Venus fluorescence turns on only when tau is assembled, ranging from soluble dimers to filamentous aggregates. By using a tau-BiFC cell model, we were able to investigate pathological tau oligomerization controlled by diverse post-translational modifications, such as glycosylation and ubiquitination (Choi et al., 2016; Han et al., 2014; Lim et al., 2015), and to identify prion-like tau species (Kim et al., 2015b). Here, we describe the tau-BiFC technique applied to a tau transgenic mouse model as a tool to monitor tau aggregation in the brain.

2. Results

2.1. Generation of transgenic mice expressing human TauP301L-BiFC

To generate the TauP301L-BiFC construct, full-length human tau (2N4R) containing a P301L mutation was fused with BiFC compartment (hTau301L-VN173 and hTau301L-VC155). P301L mutation in exon 10 of tau causes early onset tauopathy known as frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) and has been used to establish tau transgenic mouse models (Lewis et al., 2000; Santacruz et al., 2005). For neuronal expression, the neuron-specific murine Thy1.2 promoter was fused to TauP301L-BiFC construct (Feng et al., 2000) (Fig. 1A, B). The TauP301L-BiFC constructs were injected into one-cell fertilized embryo by microinjection. Heterozygous transgenic mice were screened using genomic DNA samples obtained from tail biopsies. Expression of TauP301L-BiFC constructs in the brain was confirmed using brain lysates of 3-month-old mice (Fig. 1C). Immunoblot analysis with anti-tau antibody (Tau5) indicated two bands of TauP301L-BiFC compartments of approximately 76 kDa (TauP301L-VC155) and 85 kDa (TauP301L-VN173). The expression level of human tau (hTau) was 0.34 ± 0.07-fold relative to that of endogenous mouse tau (mTau). Immunofluorescence staining of brain tissue slices further confirmed neuronal expression of TauP301L-BiFC compartment (Fig. 1D). Human tau protein was stained with anti-human tau antibody (T14), and neuronal cells were counter-stained with NeuN antibody. At three months, most neurons in TauP301L-BiFC mice were T14-positive in the cortex and the hippocampus, although the expression levels varied (Fig. 1E). The percentage of T14-positive neurons were 98.5 ± 2.6 % in the cortex and 95.2 ± 2.6 % in the hippocampus.

2.2. Evaluation of locomotor and cognitive functions in TauP301L-BiFC mice

First, to evaluate the locomotor activity of TauP301L-BiFC mice, open field test was performed with 3, 6, 9, and 12 month-old TauP301L-BiFC mice and the littermate controls. The total distance moved by TauP301L-BiFC mice was significantly decreased in 6-month-old (P < 0.01) and 9 month-old mice (P < 0.001) compared with their littermate controls (Fig. S1A). At 12 months, littersmates also showed decreased locomotor activity, and there was no significant difference between TauP301L-BiFC and wild type mice (Fig. S1A, B). Next, for the novel object recognition test, 1 day after familiarization, one of the old objects was replaced with a novel object of different color, shape, and texture (Fig. 2A). Recognition index was calculated (recognition index = time spent exploring an object / total spent time exploring objects). At 12 months, TauP301L-BiFC mice failed to distinguish the novel object from the familiar object, with a recognition index value of 0.52 ± 0.04 (Fig. 2B).

The Morris water maze test was performed to assess spatial reference memory. Mice were trained for 5 days in a water-filled pool to navigate to a hidden platform (Fig. 2C). After 5 days of training, wild type littersmates showed significantly decreased escape latency time (P < 0.001). In contrast, even after 5 days of training, TauP301L-BiFC mice showed no difference in the escape latency indicating the impaired spatial memory (Fig. 2D). On the 6th day, the hidden platform was removed, and the time spent in the quadrant of the hidden platform was measured (Fig. 2C). Littersmates spent most time in the target quadrant (TQ), whereas TauP301L-BiFC mice spent most time in the opposite (OP) and the adjacent right (AR) quadrants (Fig. 2E, F). These results suggested that TauP301L-BiFC mice failed to learn and memorize the location of the target platform. There was no significant difference between TauP301L-BiFC mice and wild type mice at 12 months for locomotor activity in the Morris water maze test (Fig. 2G). In addition, spatial learning and memory performance of TauP301L-BiFC mice was compatible to that of littersmates at 6 months (Fig. S1C, D).

To evaluate fear memory, 12 month-old TauP301L-BiFC mice and the littersmates were subjected to the passive avoidance test. On the first day of the trial, both transgenic mice and littersmates entered the dark chamber without hesitation. Upon entry into the dark chamber, an electric foot shock was delivered (Fig. 2H). On the next day, latency time to cross over to the dark chamber was measured. Most of the littersmate controls did not enter the dark chamber associated with the electric shock. In contrast, all TauP301L-BiFC mice entered the dark chamber, indicating impaired fear memory (Fig. 2I). Our results indicate significantly impaired cognitive functions of TauP301L-BiFC mice at 12 month-old.

2.3. Whole brain-image analysis of TauP301L-BiFC fluorescence

Next, we evaluated BiFC fluorescence in the brains of transgenic mice. For age-dependent analysis, 3, 6, 9, and 12 month-old TauP301L-BiFC mice were sacrificed, and whole brain slices were prepared for BiFC image analysis (Fig. 3A). Though Tau BiFC fluorescence was detectable from young animals (Fig S2), here we characterized TauP301L-BiFC mice from 3 months of age, at which brain maturation is complete (Hammelrath et al., 2016; Ingálahliikar et al., 2014). At 3 months,
extensive BiFC fluorescence was observed throughout the brains of TauP301L-BiFC mice (Fig. 3A). High-magnification images show that tau-BiFC fluorescence enriched in the somas and axons of cortical and hippocampal neurons at 12 months (Fig. 3B). BiFC fluorescence was significantly decreased by 6 months; 0.3 ± 0.1-fold in the somatosensory cortex, 0.3 ± 0.1-fold in the motor cortex, 0.2 ± 0.2-fold in the visual cortex, and 0.4 ± 0.1-fold in the entorhinal cortex, compared to 3 months (Fig. 3C). BiFC fluorescence increased at 12 months; 2.1 ± 0.2-fold in the somatosensory cortex, 1.7 ± 0.2-fold in the motor cortex, 1.6 ± 0.1-fold in the visual cortex, and 2.2 ± 0.4-fold in the entorhinal cortex, compared to 3 months (Fig. 3C). In contrast, the hippocampus showed an age-dependent increase in BiFC fluorescence intensity. The greatest increase was observed in the CA1 region, which demonstrated a 2.9 ± 0.4-fold increase at 12 months compared to 3 months. The CA3 and dentate gyrus (DG) showed comparably low BiFC fluorescence compared to CA1 region (Fig. 3C). Among other brain regions, the amygdala showed the strongest BiFC fluorescence, with an increase in intensity by 1.7 ± 0.2-fold at 12 months compared to 3 months.

Tau phosphorylation levels were investigated using immunofluorescence analysis with anti-p-TauS202/T205 (AT8) antibody, which detects late-stage tau aggregates (Augustinack et al., 2002). In the somatosensory cortex, strong BiFC fluorescence was observed in the neuronal soma at 3 months. At 6 months, BiFC fluorescence was decreased. From 9 months, BiFC fluorescence was increased in soma and axons of somatosensory neurons (Fig. 3D). In hippocampal neurons, TauP301L-BiFC fluorescence intensity increased in an age-dependent manner. At 12 months, substantial BiFC fluorescence was observed in the CA1 region (Fig. 3D). In the cortex and hippocampus, AT8-positive neurons were observed from 9 months; the numbers were higher at 12 months (Fig. 3D). The merged images of Tau-BiFC and AT8 suggest that TauP301L-BiFC oligomers formed in the early stages and promote tau hyperphosphorylation, resulting in the accumulation of AT8-positive neurons. Confocal image analysis revealed co-localization of AT8-positive neurons with TauP301L-BiFC-positive neurons at 12 months (Fig. S4). AT8-positive neuron was not detected in the brains of littermate controls at 12 months (Fig. S5A, B). Whole-brain image analysis indicated that P301L-induced tau pathology started early from 3 months with observable BiFC fluorescence, and tau pathology progressed further at 9 months as exhibited by AT8-positive neurons. The decrease in cortical BiFC fluorescence at 6 months warrants further investigation.

In addition, we evaluated neuronal cell loss in transgenic mice using cresyl violet stain (Fig. 3D). Arrows indicate shrunken neurons in the somatosensory cortex and hippocampal CA1. In the somatosensory cortex of TauP301L-BiFC mice, 9.8 ± 0.2 % (P < 0.05) neuronal cell loss was observed at 12 months, while littermates did not show any noticeable change (Fig. S5C). In the hippocampal CA1, in which the strongest BiFC fluorescence was detected, 8.1 ± 0.9 % (P < 0.05) neuronal cell loss was observed at 12 months, compared to that of littermates (Fig. 3D, F).

The progression of tau pathology, represented by tau filament formation, was further confirmed by transmission electron microscopy. At 18 months, tau filaments showing the thickness of 10.9 ± 1.4 nm were observed in the cytoplasm and the axon in the cortical and hippocampal neurons (Fig. 4A–F). The results clearly indicated the progression of tau pathology from soluble oligomers to filamentous aggregates in TauP301L-BiFC mouse model.

2.4. Analysis of whole-brain lysates for pathological tau phosphorylation and aggregation

To examine the progression of tau pathology in transgenic mice, we prepared RIPA-soluble and insoluble brain lysates from 3, 6, 9, and 12 month-old TauP301L-BiFC mice and their wild-type littermates. Immunoblot analysis with total tau antibody (Tau5) indicated two fragments of human TauP301L-BiFC fragments of approximately 76 and 85 kDa, and endogenous mouse tau of approximately 52 kDa (Fig. 5A).
The expression levels of endogenous mouse tau were not significantly altered in the soluble fraction during the 1-year aging period. In contrast, levels of human TauP301L-BiFC proteins were altered. At 6 months, the full-length TauP301L-BiFC compartments were decreased by 0.5 ± 0.0-fold compared to that of 3 month-olds. Instead, low molecular weight (35−42 kDa and 17 kDa) fragments were increased in the soluble fraction (indicated by the asterisks, Fig. 5A, D). In contrast, the total amount of mouse tau (56 kDa) was not altered at 6 months. Indeed, the low molecular weight tau fragments were positive to human-specific tau antibody rather than mouse-specific tau antibody (Fig. S6). These results suggest that the exogenously expressed human TauP301L was cleaved at 6 months, generating low molecular weight tau fragments.

When tau assembles into oligomers or fibrils, the tagged N- and C-terminal compartments of Venus (VN173 and VC155) form a mature Venus fluorescent protein. Next, we isolated the mature Tau-BiFC complexes in the soluble fraction using GFP-trap® (Fig. 5B). GFP-trap® isolated only matured tau-BiFC complexes, not each Tau-VN173 or Tau-VC155 compartments (Fig. S7) (Croucher et al., 2016). The amounts of mature TauP301L-BiFC oligomers together with insoluble TauP301L-BiFC aggregates were correlated with TauP301L-BiFC fluorescence intensity in the brain. These results indicated that tau assembly occurs even at 3 months, corresponding to BiFC fluorescence intensity at 3 months (Fig. 5B). In concordance with the decreased level of BiFC fluorescence at 6 months (Fig. 5A), the level of mature TauP301L-BiFC complexes was significantly reduced in the soluble fraction, with 84.1

Fig. 2. Evaluation of cognitive function in TauP301L-BiFC mice. (A) Behavioral scheme of novel objective recognition test. Learning and memory ability of TauP301L-BiFC mice and wild-type (WT) littermates were measured at 3, 6, 9, and 12 months (WT and TG, n = 6 for 3 months, n = 15 for 6 months, n = 9 for 9 months, and n = 10 for 12 months). The mice were exposed to two identical objects (O, old). After 24 h, one object was replaced with a different object (N, novel). The mice were allowed to freely explore the two different objects. (B) Recognition index (RI) of two objects. RI was defined as the ratio between the times spent exploring the familiar object or novel object divided by total exploratory time. The recognition index close to 0.5 (dashed line) indicates mice could not discriminate novel and familiar objects. Two-way repeated measures ANOVA with Bonferroni’s multiple-comparisons test was performed. (C) Behavioral scheme of water maze test. The cross in the circle represents imaginary quadrant borders. The yellow circle indicates the hidden platform position during training sessions on days 1-5. An empty circle indicates the removed platform position during the probe trial on day 6. Maximum trial time was 60 s. (D) Escape latency during training sessions. Two-way ANOVA with Bonferroni’s multiple-comparisons test was performed; **p<0.01; n.s., nonsignificant, compared with day 1. (E) Spatial histograms of mouse positions during a probe trial. (F) Distance traveled during a probe trial. Two-way ANOVA with Tukey’s multiple-comparisons test was performed; **p<0.01, compared with TQ quadrant. (G) Time spent in each quadrant during a probe trial. A two-tailed t-test was performed. (H) Behavioral scheme of passive avoidance test. During training, each mouse received a foot shock (3 mA, 2 s) upon entry into the dark compartment. Retention test was performed 24 h after training. (I) Step through latency time to enter the dark compartment during the retention test. A two-tailed t-test was performed; ***p<0.001; *** p<0.001***.
± 0.1 % decrease at 6 months \( (P < 0.001) \) (Fig. 5E). These results suggested that the complemented human TauP301L-BiFC oligomers were cleaved in the brain, resulting in significantly reduced BiFC fluorescence at 6 months. The amount of mature BiFC oligomers did not increase at 9 or 12 months compared with that at 3 months. Instead, insoluble tau aggregates were observed from 9 months (Fig. 5C, F). In the insoluble fraction, human TauP301L-BiFC fragments were aggregated with endogenous mouse tau, suggesting that exogenously expressed human TauP301L protein activated tau pathology in the mouse brain.
Tau phosphorylation levels were evaluated using anti-p-TauS199, anti-p-TauS396, and anti-p-TauS202/T205 antibodies. In the soluble fraction, substantial levels of tau phosphorylation at S199 were observed from 3 months to 12 months in tau transgenic mice (Fig. 5G). The level of mouse tau phosphorylated at S199 was 1.5 ± 0.1-fold higher in tau transgenic mice than in age-matched controls (Fig. 5I), implying that the expression of human TauP301L mutation induced constitutive phosphorylation of mouse tau at Ser199. In contrast, the level of pS396 increased age-dependently in both exogenously expressed human tau and mouse tau, indicating time-dependent progression of tau pathology in the mouse brain (Fig. 5G, J). When we compared the major three tau kinases, GSK-3β and CDK5 activation was observed from 6 months, and ERK activation was observed from 12 months in TauP301L-BiFC mice (Fig. S8).

Insoluble tau aggregates comprising human TauP301L-BiFC compartments and fragments, as well as mouse tau, were observed from 9 months. These results suggested that exogenously expressed human tau co-aggregated with mouse tau (Fig. 5C, F). The insoluble human and mouse tau proteins showed pS202/pT205 immunoreactivity, indicating tau hyper-phosphorylation (Fig. 5H, K). Further, pS202/pT205-positive low molecular-weight tau fragments were observed in the RIPA-insoluble fraction at 9 and 12 months. Sarkosyl fractionation is a more sensitive method to separate high-order of tau aggregates (Forest et al., 2013). Sarkosyl fractionation analysis also confirmed the formation of insoluble tau aggregates composed of human and mouse tau from 9 months (Fig. S9). Whole brain analysis of TauP301L-BiFC mice presented age-dependent, pathologic changes of tau: tau oligomerization at 3 months, tau fragmentation at 6 months, and AT8-positive tau aggregation at 9 months.

2.5. Methylene blue reduced TauP301L-BiFC fluorescence responses in the brain

Next, we applied TauP301L-BiFC mice to evaluate methylene blue (MB) on inhibiting tau oligomerization. MB is known as a dirty drug, which has multiple targets affecting diverse cellular mechanism related with tau pathology. MB treatment attenuated synaptic toxicity and tau phosphorylation in tauopathy model by inhibiting tau kinases (Sun et al., 2016). MB treatment also attenuated tauopathy by autophagy activation (Congdon et al., 2012), and rescued early cognitive deficit through the regulation of proteasome activity (Medina et al., 2011). Affecting multiple targets simultaneously, MB might regulate tau pathology in multiple stages from the early phosphorylation to degradation. Nevertheless, MB is the most studied tau aggregation inhibitor that

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**Fig. 3. Whole brain-image analysis of TauP301L-BiFC fluorescence.** (A) BiFC-fluorescence images of whole brain sections from 3, 6, 9, and 12 month-old TauP301L-BiFC mice. The brain tissue sections were selected at 0.50 mm, −1.82 mm, and −3.28 mm from bregma. Scale bar, 2 mm. (B) Representative images of BiFC fluorescence in 12 month-old TauP301L-BiFC mouse cortical and hippocampal region. Scale bar, 50 μm. (C) Quantification of regional BiFC-fluorescence intensity in TauP301L-BiFC mouse brains (TG, n = 6). The mean BiFC fluorescence intensities were measured in eleven brain regions and normalized by the intensity of Habenular (Fig S2). One-way ANOVA with Dunnett’s multiple-comparisons test was performed. (D) Representative images of BiFC fluorescence, tau immunofluorescence with AT8 antibody, and cresyl violet stain in TauP301L-BiFC mouse cortical and hippocampal regions. Black arrows indicate dead neurons with shrinkage. Scale bar, 25 μm and 50 μm. (E) Quantification of AT8-immunofluorescence intensities in somatossensory cortical and hippocampal CA1 regions of TauP301L-BiFC mice (TG, n = 4). The intensities were calculated using Image J. One-way ANOVA with Tukey’s multiple-comparisons test was performed; **p<0.01, ***p<0.001, compared with 3 months. (F) Quantification of somatossensory cortical neurons and hippocampal CA1 neurons from 3, 6, 9, and 12 month-old TauP301L-BiFC mice and wild-type (WT) littermates (WT and TG, n = 4). Two-way ANOVA with Tukey’s multiple-comparisons test was performed; *p<0.05, compared with 3 months; # p<0.05, compared with 12 month-old WT mice.

**Fig. 4. Transmission electron microscopy of tau filaments in the brain of TauP301L-BiFC mice.** Representative electron microscopy images of tau filaments in 18 month-old TauP301L-BiFC mouse. (A) and (B) show tau filaments in the soma of cortical neurons and the (D) and (E) show the magnified images. Nu, nucleus; Mt, mitochondria. (C) shows tau filaments in the axon of a hippocampal neuron and (F) shows the magnified image. Scale bar, 1000 nm for A–C; 250 nm for D–F.
Fig. 5. Analysis of whole-brain lysates for pathological tau phosphorylation and aggregation. (A–C) Immunoblot analysis of total tau with Tau5 antibody. The total tau level in RIPA-soluble (A), GFP-trap (B), and RIPA-insoluble (C) fractions of 3, 6, 9, and 12-month-old TauP301L-BiFC mouse brain extracts were compared to 12-month-old wild-type (WT) mice. Green arrows indicate hTau-VN173 and hTau-VC155. Black arrows indicate endogenous murine tau. Bands below 56 kDa show tau fragments. (D–F) Quantification of total tau protein in RIPA-soluble (D), GFP-trap (E), and RIPA-insoluble (F) fractions (WT and TG, n = 4). The relative amounts of total tau were quantified by ImageJ. One-way ANOVA with Tukey’s multiple-comparisons test was performed; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, compared with 3 months. (G–H) Immunoblot analysis of phosphorylated tau with anti-pTau\textsuperscript{S199}, anti-pTau\textsuperscript{S396}, and anti-p-Tau\textsuperscript{Ser202/T205} (AT8) antibodies. The phosphorylation level of tau protein in RIPA-soluble (G) and RIPA-insoluble (H) fraction of brain extracts from 3, 6, 9, and 12 month-old TauP301L-BiFC mice were compared to 12 month-old WT mice. (I–K) Quantification of soluble tau phosphorylation and insoluble tau hyper-phosphorylation (WT and TG, n = 4). The relative amounts of phosphorylated tau were quantified by ImageJ. One-way ANOVA with Tukey’s multiple-comparisons test was performed; *p < 0.05, **p < 0.01, ****p < 0.0001, compared with 3 months; ***p < 0.001, compared with 12 month-old TauP301L-BiFC mice.
reduces insoluble tau aggregation preventing memory deficits in diverse mouse models of tauopathy (Hochgräfe et al., 2015; O’Leary et al., 2010). A number of in vitro study have showed that MB inhibits tau aggregation from the early oligomerization stage (Taniguchi et al., 2005). However, its inhibitory activity on tau oligomerization has not been carefully evaluated in vivo models of tauopathy. Violet et al. reported that MB inhibits tau oligomerization and DNA damages in hyperthermia-induced model by using oligomer-specific T22 antibody (Violet et al., 2015). To evaluate the effect of MB on tau oligomerization in the context of tau pathology, MB was administered to the TauP301L-BiFC mice for 4 months from 8 to 12 months of age (Fig. 6A). Congruently to the previous studies, MB-treatment attenuated recognition...
memory deficits of TauP301L-BiFC mice by showing a recognition index value of 0.58 (P < 0.05) in the novel object recognition test (Fig. 6B). MB-treatment also attenuated learning impairment of TauP301L-BiFC mice by showing an improved learning performance over vehicle-treated mice during Morris water maze acquisition (day 4, P < 0.05). Although the improved learning behavior was not observed on day 5, MB-treated mice exhibited a significantly higher preference for the target quadrant than vehicle-treated mice on probe tests (P < 0.01) (Fig. 6C, D).

Then, the brains were extracted for the evaluation of tau oligomerization and phosphorylation. MB-treated group showed a significantly decrease of BiFC fluorescence, showing 31 ± 4% decrease in the cortex and 43 ± 19 % decrease in hippocampus. Corresponding to the BiFC responses, a significant decrease of AT8-immunoreactivity was observed in the cortex (35 ± 9%) and hippocampus (49 ± 8%) (Fig. 6E, F). TauP301L-BiFC-positive neurons and AT8-positive neurons were decreased in the somatosensory cortex and the hippocampal CA1 of the MB-treated mice, compared to that of vehicle-treated group (Fig. 6E, F). Then, we evaluated the neuroprotective effect of MB in the cortex and hippocampus regions of TauP301L-BiFC mice with Cresyl violet stain. In MB-treated mice, the number of neurons were increased 9.6 ± 0.3 % in the somatosensory cortex and 7.7 ± 0.5 % in the hippocampal CA1, but the results were not statistically significant due to the individual variation.

The brain lysates were also prepared to evaluate tau phosphorylation and aggregation. In MB-treated group, the level of pS199-tau decreased by 27.8 ± 4.8 % and the level of pS396-tau decreased by 43.6 ± 5.9 % in the human forms (Fig. 6G, H). The levels of pS202/T205-tau decreased by 53.7 ± 6.6 % in the RIPA-insoluble fraction of MB-treated mice (Fig. 6I, J). To evaluate the level of matured Tau-BiFC complexes in the brain lysates, GFP-Trap® assay was followed. The level of soluble tau-BiFC complexes decreased by 41 ± 12 % in MB-treated group (Fig. 6K, L), reflecting the decreased BiFC fluorescence in the brain tissue. The results indicated that MB-treatment suppresses tau phosphorylation, oligomerization and aggregation in TauP301L-BiFC model.

3. Discussion

It is becoming clear that soluble tau oligomers play a key role in tau pathology as a neurotoxic species causing neuronal cell death and also as a prion-like seed mediating tau propagation. Due to the pathological implications, tau oligomers are considered to be an important therapeutic targets to prevent tauopathies including Alzheimer’s disease. Accumulating studies have reported potential drug candidates inhibiting tau oligomerization. However, those inhibitory effects were evaluated in vitro by using purified tau and aggregation inducers, not in vivo models of tauopathies due to the difficulty of characterizing tau oligomers in the brain. Here we present a transgenic TauP301L-BiFC mouse model that can visualize tau self-assembly from soluble oligomers to insoluble aggregates. By introducing BiFC techniques to tau, we could achieve spatial and temporal resolution of tau assembly in the brain. In the brain of TauP301L-BiFC mice, soluble TauP301L-BiFC complexes were formed even at 3 months of age, showing a significant BiFC-fluorescence. The intensive maturation of TauP301L-BiFC in the brain correlated with the previous in vitro studies showing that tau containing P301L mutation favors the formation of oligomers (Guo and Lee, 2011; Kim et al., 2015a; Waxman and Giasson, 2011).

The maturation of BiFC complex is irreversible, therefore we expected that tau aggregation process would be facilitated by the stable TauP301L-BiFC complexes in the brain (Kerppola, 2008). Instead, significantly decreased tau-BiFC fluorescence was observed at 6 months of age, with presenting massive tau fragmentation. Tau fragmentation is known to be part of the conserved pathologic process, generating ‘neurotoxic’ truncated tau that accelerates neuronal cell death and insoluble tau aggregation (Cotman et al., 2005; Ferreira, 2012; Mahaman et al., 2018). Indeed, insoluble tau aggregates composed of human and

Table 1

Pathological features of TauP301L-BiFC mouse model.

| TauP301L-BiFC | 3 month | 6 month | 9 month | 12 month |
|--------------|--------|--------|--------|--------|
| Tau conformation | Soluble oligomers | + + + | + | + | + |
| Insoluble aggregates | – | – | + | + | + |
| Cleaved form | – | – | + | + | + |
| Tau phosphorylation | pS199 | + + | + | + | + |
| | pS396 | + | + | + | + |
| | pS202/T205 | – | + | + | + |
| Neuronal cell loss | Hippocampus (CA1) | – | – | + | + |
| | Somatosensory Cortex | – | – | – | + |
| Cognitive Deficits | Novel object recognition | Not failed | N.A. | N.A. | Failed |
| | Water maze | N.A. | N.A. | N.A. | Failed |
| | Passive avoidance | N.A. | N.A. | N.A. | Failed |

The level of expression and severity of specified phenotypic traits are represented by the proportional number of signs (i.e., negative (−), low (+), moderate (++), severe (+++), extreme (++++)). n.a., not analyzed.)
mouse tau were observed from 9 months of age, suggesting that human tau fragments promote the aggregation of mouse tau (Table 1). Also from the 9 months, AT8-positive neurons were accumulated in the cortex and hippocampus. AT8 has been considered as a late stage marker, recognizing pre-neurofibrillary tangle (pNFT), intracellular tangle (iNFT), and extra-neuronal neurofibrillary tangle (eNFT) (Biernat et al., 1992). However, AT8 immuno-reactivity was also reported in human brain tissue prior to the development of fibrillary pathology (Stratmann et al., 2016). Therefore, the progression of tau pathology, which is represented by the formation of tau fibrils, needs to be confirmed using electron microscopy. In TauP301L-BiFC mice, we could observed enriched tau filaments (10.9 ± 1.4 nm, thickness) in cortical and hippocampal neurons at 18 months.

Hence, our TauP301L-BiFC mouse model provides advantages compared to other tau transgenic mouse models. First, in comparison to P301L tau transgenic mouse model, our transgenic mouse model improves the visualization of tau dynamics by conjugating BiFC to tau while it does not promote or delay insoluble tau aggregation. As similar to P301L tau transgenic mouse model that develops insoluble tau inclusions at the age of 8–9 months (Götz et al., 2001; Terwel et al., 2005), our TauP301L-BiFC mouse model exhibits similar tau-dependent pathology with enhanced tau resolution. Second, in comparison to rTg4510 and JNPL tau transgenic mouse lines expressing short tau isoforms (0 N 4R), our full-length TauP301L-BiFC mouse model presents the early stages of tau pathology such as oligomerization, proteolytic cleavage, and nucleation reaction. Currently, the most widely used tau transgenic mouse lines such as rTg4510 and JNPL3, express a short tau isoform, to reduce time to develop tau pathology (Lippens et al., 2007). Tau fragments containing microtubule-binding repeat domains act as seeds to accelerate nucleation reaction for tau polymerization. rTg4510 mice developed hyperphosphorylated insoluble tau even at 4 months with cognitive deficits (Ramsden et al., 2005). JNPL3 mice developed neurofibrillary tangles at 4.5 months with motor behavioral deficits (Lewis et al., 2000). Through the over-expression of aggregation prone form, tau aggregation occurs much faster in these models. Accordingly, it is not easy to figure out the early stages of tau-dependent pathology. In this context, our full length TauP301L-BiFC mouse model may be a feasible tool to better understand the early stage of tau-associated pathology.

In tau-BiFC mice, cognitive deficits were observed at 12 month of age, with significant neuronal cell death. In this study, to avoid hormonal effect on the behavioral activity, we performed all behavioral tests in male mice. In rTg4510 mouse model, females showed significantly higher levels of hyperphosphorylated tau than males (Yue et al., 2011). Also, in APP/PS1/tau triple transgenic mouse model, female displayed more prominent amyloid plaques, neurofibrillary tangles, neuroinflammation, and spatial cognitive deficits than male (Yang et al., 2018). In this regard, it will be interesting to carefully characterize the sex difference of behavioral phenotypes in the future study. Also, unbiased stereological analysis is needed for the future study to obtain accurate and precise estimates of the number of cells in whole brain regions (Mouton, 2013; West, 2013). In this study however, neuronal cell densities were analyzed only in 30 μm-thick tissue sections, which not allowing the estimation of total number of neurons in the whole brain area.

On the other hand, we consider that an experimental limitation of the TauP301L-BiFC mouse model is occurred due to the autofluorescence of brain tissue in aged animals. To examine BiFC fluorescence efficiently in aged mice, it is necessary to quench the background autofluorescence. In order to circumvent this limitation, we suggest Sudan black B staining that is useful to quench the autofluorescence. Also, BiFC fluorescence intensity does not provide any structural information of tau aggregates. To verify the presence of tau fibrils, ultrastructural analysis at different stages is required.

In summary, we developed a novel full-length tau transgenic mouse model so called TauP301L-BiFC mouse model. This mouse model apparently exhibited a series of tau pathology including proteolytic cleavage, oligomerization, and fibrillation. Moreover, TauP301L-BiFC mice showed neurodegenerative phenotypes such as enhanced neuronal damage and atrophy. Taken together, a tool to visualize tau assembly in the brain, TauP301L-BiFC transgenic mouse model will be a useful and feasible tool for studying tau pathology and developing therapeutics targeting tau oligomers.

4. Methods

4.1. Generation of TauP301L-BiFC transgenic mice

To generate TauP301L-BiFC transgenic mice, cDNA constructs of hTauP301L-VN173 and hTaup301L-VC155 were cloned into the murine Thy1 promoter cassette (pTSC21K) at Xho1 site (Kong et al., 1992). After linearizing the transgene vectors with EcoRI enzyme, the purified transgene fragments were microinjected into fertilized embryos from C57BL/6 mice and were transplanted into pseudo-pregnant females in Macrogene, Inc. One founder from the injections was identified by PCR of genomic DNA extracted from tail biopsy using standard methods of proteinase K digestion, high salt precipitation, and ethanol precipitation (Miller et al., 1988). The presence of the TauP301L-BiFC transgene was identified with the following sets of primers: PCR primers for Thy1-hTaup301L-VN173, forward 5′-CACATGAGCCACGCTCTCTCA-3′ and reverse 5′-TGACTGTACGAGTACAGACGAT-3′; and for Thy1-hTaup301L-VC155, forward 5′-GAGAAGCGGATCAGTGT-3′ and reverse 5′-AGTACAGGCGACGGTCC-3′. Founder animal was crossed with C57BL/6 mice to establish lines. Transgenic offspring were interbred with their wild type littermates and maintained as heterozygotes. Mice were housed on a 12:12 h light-dark cycle in pathogen-free facilities at the Korea Institute of Science and Technology. Animal protocols followed the principles and practices outlined in the approved guidelines by the Institutional Animal Care and Use Committee of the Korea Institute of Science and Technology. All animal experiments were approved by the Korea Institute of Science and Technology.

4.2. Behavioral tests

To evaluate the effects of tau pathology at a behavioral level, four different age groups (3, 6, 9, and 12 month-old) TauP301L-BiFC mice (n = 6 for 3 month-old group, n = 15 for 6 month-old group, n = 9 for 9 month-old group, and n = 10 for 12 month-old group) were used in the open field and novel object recognition tests. Twelve-month-old TauP301L-BiFC mice and littermate controls were tested in the Morris water maze and passive avoidance tests. Behavioral testing was performed between 10:00 a.m. and 6:00 p.m. Mice were habituated to the procedure room for 2 h at the beginning of each test. All tested animals were male. In each individual test, TauP301L-BiFC mice were compared with littermate controls (n = 6 for 3 month-old group, n = 15 for 6 month-old group, n = 9 for 9 month-old group, and n = 10 for 12 month-old group).

4.2.1. Open field test

General activity and locomotion were assessed in an open field arena (40 × 40 cm) with black walls and a white floor. Mice were individually placed in the center and allowed to freely explore for 15 min. The total distance moved and travel pathways were automatically recorded and analyzed using Noldus EthoVision XT video tracking system.

4.2.2. Novel object recognition test

Novel object recognition test was performed as described previously (Leger et al., 2013). Briefly, in the training trial (familiarization phase), mice were allowed to explore two identical objects in an open field, to which they had been habituated, for 10 min. In the testing trial (recognition phase) performed 24 h later, one familiar object was changed.
for a novel object, which was different in color and shape. Mice were allowed to explore the objects for 10 min. The exploration time for the familiar (old) or the new object (novel) during the test phase was recorded using Noldus EthoVision XT video tracking system. Memory was operationally defined by the recognition index calculated by dividing the time an animal spent exploring the novel object or familiar object by the total time spent exploring objects in the testing period. The exploration time measured when the mouse was pointing towards the object in the vicinity of the object.

4.2.3. Morris water maze test

Morris water maze test was conducted based on a standard protocol described by Vorhees et al. (Vorhees and Williams, 2006) with the following modifications. The maze comprised a large swimming pool (155 cm in diameter) filled with opaque water (22 ± 2 °C) that dissolved non-toxic white paint. Five pre-training sessions consisting of four trials per day were performed on day 1–5 to find the hidden platform (15 cm in diameter). Mice facing the outer edge of the pool were released from four different starting positions and allowed to explore the platform for 60 s. Thereafter, the mouse was guided to the platform by the experimenter. The sixth trial session on day 6 was performed as a probe trial. Data were collected and analyzed using Noldus EthoVision XT video tracking system. The escape latency during the pre-training sessions was compared between TauP301L-BiFC mice and littermate control groups. The difference in time spent in each quadrant relative to chance (25 %) was compared for the probe trial.

4.2.4. Passive avoidance

Passive avoidance test was performed as described previously (Van der Poel, 1967) with the following modifications. Mice were acclimated in the passive avoidance chamber (Gemini) for 10 min and were then returned to their home cages. The chamber consisted of lit and dark compartments separated by a gate. The following day, mice were placed in the lit compartment, and the gate was opened after 30 s. When mice crossed to the dark compartment, the gate was closed and an electrical foot shock (3 mA, 2-second duration) was applied. Mice were left in the dark compartment for 30 s after the foot shock to enable association of the environment with the aversive stimulus. Mice were then returned to their home cages. The following day, mice were placed in the lit compartment again, and the gate was opened after 30 s. The time required for mice to enter the dark compartment was measured as the latency with a maximum period (cut-off time) of 540 s. Mice were subsequently removed and returned to their home cages.

4.3. Brain tissue preparation

Mice were perfused with 0.9 % saline and fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde. Then, brains were rapidly extracted and fixed with PBS containing 4% paraformaldehyde for 48–72 hr. For cryoprotection, the brains were transferred to PBS containing 30 % sucrose solution and incubated at 4 °C until they sunk. For cryosectioning, the brains were embedded with O.C.T (Tissue-TEK, USA). The samples were cut serially as 30-μm thick coronal sections on a cryostat (Leica). Brain tissue slices were transferred to PBS containing 0.05 % sodium azide and preserved at 4 °C.

4.4. Sudan Black B stain and BiFC image acquisition

Brain tissue sections (n = 6 each for 3, 6, 9, and 12 month-old TauP301L-BiFC mice and littermate controls) were mounted onto slides. To reduce autofluorescence, brain tissue sections were stained with Sudan Black B solution (70% ethanol containing 0.05 % Sudan Black B) for 10 min, washed three times with PBS containing 0.1 % Triton X-100, and washed with distilled water. For counter-staining nuclei, brain tissues were stained with Hoechst (0.5 μg/mL dissolved in distilled water) for 30 min. BiFC fluorescence (λex = 460–490 nm and λem = 500–550 nm) images of whole brain tissues were acquired using Operetta (PerkinElmer) and Axio Scan. Z1 (Zeiss). High magnification images of Tau-BiFC fluorescence were acquired using Nikon confocal microscope with 100X objective lens.

4.5. Quantification of Tau-BiFC fluorescence in the brain

Mean BiFC-fluorescence intensity was measured in eleven brain regions from: somatosensory motor, visual, and entorhinal cortices, hippocampal CA1, CA3, and dentate gyrus (DG), amygdala, thalamus, and striatum. Motor cortex and striatum were obtained from AP: 0.50, Somatosensory cortex, hippocampal CA1, CA3, and DG, thalamus, and amygdala were obtained from AP: -1.82. Visual and entorhinal cortices and subiculum were obtained from AP: -3.28. (Fig. S2). To normalize the background fluorescence between the brain samples, habenular region was used as an internal control. Using image J software (NIH), the region of interest was masked, and the fluorescence intensity value was calculated. Data were shown as the average and standard deviation of Mean fluorescence intensities of six brain images from six animals.

4.6. Transmission electron microscopy

18 month-old TauP301L-BiFC mouse was perfused with 0.9 % saline and then, brain was rapidly extracted. Thick coronal sections of 0.3 mm containing hippocampus and cortex were cut with a vibratome (Leica VT1200, Leica Microsystems, Austria). The tissues were then postfixed with 1% OsO4 dissolved in 0.1 M PB for 2 h and dehydrated in ascending gradual series of ethanol and infiltrated with propylene oxide. Specimens were embedded by Poly/Bed 812 kit (Polysciences). After pure fresh resin embedding and polymerization at 65°C electron microscope oven (TD-700, DORAKA, Japan) for 24 h, sections were initially cut about 70 nm thick by LEICA EM UC-7 (Leica Microsystems, Austria) with a diamond knife Diatome and transferred on copper and nickel grids. Sections were then double stained with 6% uranyl acetate EM5, 22,400 for 20 min and lead citrate (Fisher) for 10 min for contrast staining. Sections were observed by transmission electron microscopy (JEM-1011, JEOL, Japan) at the acceleration voltage of 80 kV. Images were acquired using Camera-Megaview III (Soft imaging system, Germany).

4.7. Immunofluorescence analysis

For immunofluorescence image analysis, brain tissue slices (n = 6 per group) were stained with T14 (hTau, 1:200, ThermoFishier) and NeuN (1:500, Millipore) antibodies. The primary antibodies were detected using Cy5-, or Alexa568-conjugated anti-mouse or anti-rabbit secondary antibodies. Fluorescence images (λex = 620–640 nm and λem = 650–700 nm) were acquired using Operetta (PerkinElmer).

For AT8-immunofluorescence imaging with Tau-BiFC fluorescence, brain tissue slices were stained with AT8 (p520/2/1205, 1:200, Abcam) antibody and detected with Cy5-conjugated anti-mouse antibody. AT8-stained brain slices were mounted onto glass slides and stained with Sudan Black B solution (70 % ethanol containing 0.05 % Sudan Black B) for 10 min. The slides were rinsed with PBS containing 0.1 % Triton X-100 to remove the excessive solution. For counter-staining nuclei, brain tissues were stained with Hoechst (0.5 μg/mL dissolved in distilled water) for 30 min. BiFC fluorescence (λex = 460–490 nm and λem = 500–550 nm) and AT8-BiFC fluorescence images (λex = 620–640 nm and λem = 650–700 nm) were acquired using a Zeiss Axio Scan Z1 (Zeiss, Oberkochen, Germany).

4.8. Cresyl violet stain and Surf-Volume-Ratio analysis for neuronal cell count

Brain tissue slices (n = 6 per group) of TauP301L-BiFC mice and littermate controls were stained with Cresyl violet (do Nascimento...
et al., 2012). Brain tissue slices mounted on glass slides were immersed in 100 % ethanol for 2 min, and xylene for 15 min. Then brain tissue slices were re-hydrated in a series of graded alcohols until water is used. Then, brain tissue slices were immersed in 1% Cresyl violet Acetate solution (Sigma) for 10 min, and the slices were dehydrated with graded ethanol and with xylene. Then, the brain tissue slices were mounted with DPX mounting medium (Sigma).

For neuronal cell counting, motor cortex, somatosensory cortex, and hippocampal (CA1 and CA3) regions were imaged using Ti microscope (Nikon, 20X objective lens) and cellular morphology analysis was performed by using Surf-Volume-Ratio function of NIS-Elements BR software (Nikon). Cresyl stained cells, which have diameters >5 μm and contain a dark nucleolus within a lightly stained cytoplasm, were counted as healthy neurons. The morphological evaluation was performed on 30 μm-thick coronal sections (AP: -0.5 and AP: -1.82). The region of interest was 140 μm × 140 μm of each cortical and hippocampal area.

4.9. Preparation of RIPA-soluble and insoluble brain lysates

Mice were perfused with 0.9 % saline, and brains were extracted (n = 6 per group for 3, 6, and 9 month-old mice; n = 10 per group for 12 month-old mice). Brains were weighed and suspended in RIPA lysis buffer (Sigma) containing protease and phosphatase inhibitor cocktail. Then, tissues were disrupted using Dounce homogenizers (2 ml glass Dounce homogenizer) and incubated at 4 °C for 2 h. The homogenized mixtures were centrifuged at 20,000 g at 4 °C for 20 min. Supernatants were collected as RIPA-soluble fractions and stored at −80 °C. To prepare RIPA-insoluble fractions, the remaining pellets were washed once with RIPA lysis buffer containing 1 M sucrose resuspended in 2% SDS solution (1 ml per gram of tissue) and incubated at RT for 1 h. To obtain RIPA-insoluble fractions, the mixtures were centrifuged at 20,000 g for 1 min at RT. The supernatant was collected and preserved at −80 °C.

4.10. Preparation of sarkosyl-insoluble brain lysates

Mice were perfused with 0.9 % saline, and brains were extracted (n = 2 per group for each ages). Brains were weighed and suspended in ice-cold low salt (LS) buffer (50 mM HEPES pH 7.0, 250 mM sucrose, 1 mM EDTA) containing protease and phosphatase inhibitor cocktail. Then, tissues were disrupted using Dounce homogenizers (2 ml glass Dounce homogenizer). To each 0.8 ml aliquot, 100 μL each of 5 M NaCl and 10 % (w/v) sarkosyl to concentrations of 0.5 M and 1% w/v were added, respectively, and incubated on ice for 15 min. The homogenized mixtures were sonicated for three 5 s pulses at 30 °C amplitude using a microtip probe and centrifuged at 180,000 g at 4 °C for 30 min. The supernatants were transferred as sarkosyl-soluble fractions. The pellets were incubated in 200 μL of urea buffer (50 mM Tris – HCl pH 8.5, 8 M urea, 2% SDS) containing protease and phosphatase inhibitor cocktail for 30 min at room temperature. The resuspended pellets were sonicated for 1 s pulses at 20 % amplitude and stored at −80 °C as sarkosyl-insoluble fractions.

4.11. Immunoblot analysis

For immunoblot assay, 20 μg of each lysate was separated by 10 % SDS-PAGE gel and transferred to PVDF membrane. RIPA-insoluble fractions were diluted 10-fold in 2% SDS solution, and 20 μL of each sample was loaded on SDS-PAGE gel. The levels of total tau and phosphorylated tau were detected by anti-tau antibody, Tau5 (Abcam), 77G7 (BioLegend), and anti-phospho-tau antibodies, pSer199 (Abcam), pSer396 (Abcam), and AT8 (Invitrogen). Anti-actin antibody (Abcam) was used as a loading control. For the clear visualization of human and mouse tau, two different exposure times were applied for ECL detection; 1 min for mouse tau and 5 min for human tau (Fig. 4A, C, G, and H). To evaluate tau kinases enzymes, anti-pERK1/2 (Abcam), anti-ERK1/2 (Abcam), anti-pGSK3β-Y216 (Abcam), anti-GSK3β (Abcam), anti-P35/P25 (Abcam), and anti-CDK5 (Abcam) antibodies were used. Band intensity was quantified using Image J software (NIH).

4.12. Isolation of mature TauP301L-BiFC complex using GFP-Trap*

To isolate soluble TauP301L-BiFC complexes, GFP-Trap® (ChromoTek) immuno precipitation was performed using RIPA soluble brain lysates from 3, 6, 9, and 12-month-old mice. Each lysate (1 mg) was incubated with 50 μL of GFP-Trap® beads at 4 °C overnight. Then, beads were washed three times with washing buffer (10 mM Tris/Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA). Bead-bound proteins were eluted by adding 120 μL of 2X SDS sample buffer and boiled at 95 °C for 5 min.

4.13. Administration of methylene blue to TauP301L-BiFC mice

To evaluate tau aggregation inhibitor in vivo, Methylene blue (Sigma) was intraperitoneally administered to 8-month-old TauP301L-BiFC mice for 4 months, three times a week, with 10 mg/kg dosage (n = 8 per group). For control group, PBS-treated TG mice and age-matched WT mice were used. Behavioral assessments were conducted as described above. For BiFC fluorescence analysis and immunofluorescence analysis, only TG mice were used and conducted as described above.

4.14. Statistics

A two-tailed t-test was performed when two groups were compared. One-way ANOVA or two-way ANOVA was performed when multiple groups were compared, depending on the number of independent variables. Statistical analysis was performed using Excel (Microsoft Corp.) or Prism software (GraphPad Software, Inc.).

Author contributions

Y.K.K. designed the study and provided theoretical guidance; S.S., D.K., J.Y.S., H.J., S.J.H., and S.L. performed the experiments; S.S., D.K., S.L., and Y.K.K. interpreted the data and wrote the manuscript; and S.S., N.W.K., H.R., A.P., S.L., and Y.K.K. analyzed the data and revised the manuscript. All authors reviewed the final version of the manuscript.

Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. The Peer Review Overview and Supplementary data

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