Intracellular Tau Fragment Droplets Serve as Seeds for Tau Fibrils

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Abstract

Intracellular tau aggregation requires a local protein concentration increase, referred to as droplets. However, the cellular mechanism remains insufficiently understood. We expressed OptoTau, a fusion of P301L mutant tau with CRY2olig, a light-sensitive protein that forms homo-oligomers. Under blue light exposure, OptoTau increased tau phosphorylation and was sequestered in aggresomes. Suppressing aggresome formation by nocodazole formed tau granular clusters in the cytoplasm. The granular clusters disappeared by discontinuing blue light exposure or 1,6-hexanediol treatment, which can dissolve droplets, suggesting that intracellular tau droplet formation requires the microtubules collapse. Expressing OptoTau-ΔN, a species of N-terminal cleaved tau that was observed in the Alzheimer's disease brain, formed 1,6-hexanediol and detergent-resistant tau clusters in the cytoplasm by blue light stimulation. This intracellular stable tau clusters acted as seeds for tau fibrils in vitro. These results suggest that both tau droplet

formation and N-terminal cleavage are necessary for neurofibrillary tangles formation in neurodegenerative diseases.

Introduction

Tau, a microtubule-binding protein, is an intrinsically disordered protein that is expressed as six isoforms in the adult human brain. Tau prominently modulates the microtubule dynamics and stabilization of the cytoskeleton through direct association with tubulin dimers [1,2]. In tauopathies, hyper-phosphorylation of tau reduces the affinity for microtubules [3] and collapses microtubules [4 5,6]. The hyperphosphorylated tau binds together and forms fibrillar tau aggregates having β-sheet structure in cells of the central nervous system[7-10]. Recently, cryo-electron microscopy (cryo-EM) determined atomic structures of the cores of tau fibrils from patients with tauopathies such as Alzheimer's disease[11], Pick's disease[12], chronic traumatic encephalopathy[13], corticobasal degeneration[14,15], progressive supranuclear palsy[16], argyrophilic grain disease[16], and primary age-related tauopathies[16]. These reports suggest that the core structure consists of different amino acid sequences of tau in each disease, although it is limited from the microtubule-binding domain to the C-terminal domain of tau. However, it still remains unclear how highly disordered protein tau forms the ordered aggregates, tau fibrils.

In previous studies, tau oligomerization preceding tau fibril formation is an early event in the pathogenesis tauopathies[17,18 19]. Our in vitro studies with recombinant tau have shown that fibrils consist of oligomeric and granular tau oligomers[20], which were observed in the frontal cortex of Braak stage 1 patients[21]. The formation of the tau oligomers has been repeatedly reported to be involved in toxicity [22-26]. As a mechanism for tau oligomer formation, recent reports have shown that recombinant tau first undergoes liquid-liquid phase separation (LLPS) by exposure to a crowding agent and polyanions, forming condensates referred to as tau droplet [27-31]. The droplet tau transitions into aggregates with β-sheet structure by long incubation [30,31]. The process of tau aggregation has been clarified in vitro. The intracellular mechanism of tau aggregation, including tau droplet formation and transition from tau droplets to tau oligomers, remains insufficiently characterized.

The intracellular behavior of proteins is highly dynamic and heterogeneous. Tau, an intrinsically disordered protein, is highly dynamic but develops an ordered structure during the aggregation process. However, conventional analytical methods are limited to elucidate the mechanisms and the conditions of the phase transition in cells.

Arabidopsis cryptochrome 2 (CRY2) is a photoreceptor-soluble protein, that modulates circadian rhythm in plants, leading to the regulation of development and growth. The CRY2, which can undergo homo-oligomerization by blue light, is a powerful optogenetic tool for enforcing to induce intracellular condensation of target proteins by blue light in mammalian cells [32,33 34]. CRY2olig, a CRY2 mutant (E490G), has increased oligomerization capacity compared to CRY2 [32,35]. The CRY2olig clusters were dissociated by light deprivation [32]. Amyloid proteins including α-synuclein [36] and TAR DNA-binding protein of 43 kDa (TDP-43) [37,38], fused to CRY2olig, formed their oligomers and supersaturated state upon irradiation in vivo and in vitro. Thus, utilizing CRY2olig is appropriate to analyze the process from protein condensation to aggregation with β-sheet structure.

Using CRY2olig fused to tau, we investigated the conditions under which tau assembles to form intracellular droplets within the cell, and then the conditions under which the transition from tau droplets to tau oligomers occurs.

Results

Full-length P301L with CRY2olig (OptoTau) is clustering in microtubules and transported to aggresomes.

We used P301L mutant tau with CRY2olig (OptoTau; figure S1) in all experiments, because one of frontotemporal dementia with parkinsonism (FTDP)-17 mutation P301L was reported to be a tau aggregation-prone mutation [23,39]. In SDS-PAGE western blotting, OptoTau appeared at approximately 150 kDa, and when exposed to blue light, the OptoTau band showed a mobility shift (figure 1A), suggesting that OptoTau increased the phosphorylation by blue light exposure for 24 hours. This was confirmed by the elevation of tau phosphorylation at AT8 site (figure 1B left panel), S422 (figure 1B right panel) which are involved in tau aggregation [40-42], S262 (figure S2A) which is involved

in microtubule binding[43] and T217 (figure S2B) which is a biomarker of Alzheimer's disease [44,45]. The expressing tau without CRY2olig did not show blue light-dependent tau phosphorylations in cells (figure S3), suggesting that enforced clustering tau in cells stimulated tau phosphorylation machinery. In the analysis of non-reducing condition western blotting, we found significantly (9.7-fold) increased smeared tau band above 250 kDa in the blue light exposure, compared with no exposure (figure 1C). These results indicate that the clustering OptoTau leads to tau hyper-phosphorylation and oligomerization in cells.

To investigate the localization of CRY2-FL-tau clusters in the cells, we performed co-immunofluorescence staining for tau and α-tubulin. In blue light-exposed cells, OptoTau colocalized with α-tubulin and was assembled near the nucleus, while OptoTau was uniformly distributed throughout the cells along with α-tubulin in case of no light exposure under confocal microscopy (figure 1D) and reflected light microscopy (figure S4A). It is thought that the blue light-induced the OptoTau clustering structure could be aggresomes (figures 1D and S4A), which are generated near or surrounding the cell's centrosomes[46]. The protein is labeled with polyubiquitination, allowing for retrograde transport facilitated by the binding of histone deacetylase 6 (HDAC6) and dynein, a microtubule-based motor protein [46 47]. In blue light-exposed cells, OptoTau induced the increased level of ubiquitinated protein (figure 1E). Furthermore, γ-tubulin, HDAC6 and dynein, colocalized with OptoTau (figure 1F). These results suggest that clustering OptoTau migrates along microtubules to aggresomes and may be degraded. These results lead us to investigate the effect of inhibiting aggresome formation by the destruction of microtubules on tau aggregation.

Blue light exposure induced the OptoTau droplets when in inhibition of microtubule assembly.

To inhibit microtubule polymerization, Neuro2a cells expressing OptoTau were treated with nocodazole, a reversible microtubule polymerization inhibitor [48], during blue light exposure (figure 2A). As shown in Figures 2B-D, blue light exposure to nocodazole-treated cells induced an increased level of tau phosphorylation (Figure 2C) and oligomer formation (Figure 2D). Thus, the evoked process by blue light exposure, stimulation of

tau phosphorylation and tau oligomerization, was maintained even in nocodazole treatment. In analysis using fluorescence microscopy, the granular tau clusters appeared not only in the perinuclear area but also throughout the cell (figure 2E, figure S4B and figure S5). Further, both the granular tau clusters and γ-tubulin were not observed near or surrounding the cell's centrosomes (figure 2F), suggesting that microtubule destruction led the clustering tau to form membrane-free structures in the cytoplasm, but not aggresomes.

To further characterize the granular tau clustering, we turned off the blue light and counted the number of the granular tau clustering. Light off significantly decreased the number of granular tau clustering (figure 3A), which indicates that the granular tau clustering by nocodazole and blue light exposure is a reversible structure. Previous in vitro studies similarly show that tau droplets are reversible [27-31]. 1,6-hexanediol was used for dissolving protein droplets and membrane-less organelles in in vitro and in cellular studies, but it was not effective for the stable protein aggregations like tau fibrils [49-52 53]. Blue light-induced granular tau clustering completely disappeared by treatment of 1,6-hexanediol for 60 minutes (figure 3B). The accumulation of aggregated tau exhibits resistance to the anionic surfactant sarkosyl [54]. While aggregated recombinant tau as a positive control was insolubility for sarkosyl, the sarkosyl-insoluble tau was little or no detectable in the OptoTau expressing cell lysates containing the granular tau clusters (figure 3D). No difference in the amount of total tau was detected between the samples (figure 3C). Taken together, OptoTau could form a membrane-less structure with a droplet-like nature in the cell, when exposed to blue light under the condition of nocodazole treatment.

ΔN-P301L having CRY2olig is aggregated by blue light in the cytosol in cultured cells.

Microtubule disruption could form from the OptoTau clusters to intracellular droplets, but this condition could not form tau droplets to stable tau aggregation. An additional condition is required for tau aggregation formation from tau droplets. Cryo-EM studies revealed that the core of tau fibrils is composed of a common sequence, including microtubule-binding repeat domain and a part of the C-terminal domain in tauopathies [11]

[12] [13] [14,15] [16]. Deletion of the N-terminal projection domain increased with aging in tau transgenic mice, and accelerated heparin-induced tau aggregation in vitro [55]. These findings led us to investigate the effects of N-terminal deletion. We generated ΔN-P301L with CRY2olig (OptoTau-ΔN) (figure 4A). In biochemical analysis, OptoTau-ΔN expression showed tau band mobility shift and an increase of tau phosphorylation in response to blue light exposure (figure 4B). Further, an 8.3-fold increase in tau oligomerization was observed in cells exposed to blue light (figure 4C), indicating that N-terminal deletion does not affect the tau clustering process. Under fluorescence microscopy observation, tau staining did not show the granular tau clustering shape, but looks like the assembly of tau clusters (figure 4D and figure S4C). Sarkosyl-insoluble tau was significantly increased in cells expressing OptoTau-ΔN, compared with OptoTau, when exposed to blue light (figure 4E), indicating that OptoTau-ΔN has the potential to produce tau aggregation. To characterize the clustering with the light off and exposure to 1,6-hexanediol, unlike the tau droplets, OptoTau-ΔN clusters kept the stable tau clustering even in conditions under the light off (figure 4F) and 1,6-hexanediol treatment (figure 4G), suggesting that N-terminal deletion enhances tau-tau interaction, leading to the formation of tau droplets to stable tau aggregation. Next, we investigated the shape of OptoTau-ΔN cluster formation after blue light exposure. At 1h after blue light exposure, circular-shaped tau clusters appeared in the cytoplasm. Their circularity was measured in each cluster. At 1 h after blue light exposure, the circularity of tau clusters was mainly 1.0 (figures 4H and 4I). Longer exposure to blue light resulted in the reduction of the circularity of tau clusters. (figures 4H and 4J). Tau clusters formed by short exposure were solubilized by 1,6-hexanediol (figure 4I), whereas the non-spherical tau clusters formed by long exposure were resistant to 1,6-hexanediol (figure 4J). These suggest that, over time, OptoTau-ΔN clusters convert from a droplet-like to a solid-like state of tau aggregation in response to blue light exposure, because while the shape of the liquid phase is generally governed by surface tension and becomes spherical [56-58], the shape of the solid phase such as aggregated is non-spherical [59,60].

To characterize the solid-like state of tau clusters, we examined the cell lysate by sucrose density gradient centrifugation. This method can separate tau aggregated samples from mouse [61] and human brains [21], as well as recombinant tau [20] into fractions based on

their aggregate size and composition, Fraction1: no apparent aggregates, Fraction 2: small tau granules, Fraction 3: granular tau oligomers, Fractions 4-6: short and long tau fibrils. Cell lysate expressing OptoTau-ΔN after treatment with nocodazole and blue light exposure was fractionated by sucrose density gradient centrifugation. Tau levels in fractions 2 (figure 5B) and 3 (figure 5C) were significantly increased in cells expressing OptoTau-ΔN by blue light, compared with no irradiation, but not in fractions 1 (figure 5A), 4 (figure 5D), and 5 (figure 5E). These demonstrated that aggregates from OptoTau-ΔN under microtubule destruction consist of small tau granules and granular tau oligomers which are detergent insoluble[20]. It has been reported that the phosphorylated high-molecular-weight tau is responsible for tau propagation [62], and we observed its increase in the cells co-exposed to blue light and nocodazole (see figure 4B and C). To test the promotable effect of the blue light-induced insoluble tau oligomers on tau seeding activity, a seed amplification assay [63,64] was carried out. Recombinant tau aggregation having a β-sheet structure was significantly increased by treatment of lysates from blue light-irradiated cells, compared with no irradiation (figure 5F). Atomic force microscopy (AFM) has been used in numerous studies to visualize tau aggregated structures including granular tau oligomers and tau fibrils [20,21,65,66]. The number of recombinant tau aggregates formed by blue light-exposed cell lysates was significantly higher than that by W/O light (figure 5G). The results indicate that ΔN-P301Ltau-formed oligomers can augment tau seeding activity.

Discussion

Several cellular models of tau aggregation induced by seeded tau exposure have been developed [67 68 69 70 71 72]. However, the cellular mechanism and tau aggregation pathway that convert normal tau to seed-active tau remain unclear. The study investigated the P301Ltau-mediated aggregation pathway in cultured cells and presents a novel mechanism of aggregation pathway, which includes the formation of intermediate aggregates with seeded activity. Firstly, we show that OptoTau is clustered in the aggresomes upon exposure to blue light. Secondly, we find that OptoTau is scattered to the cytoplasm and forms liquid-phase tau condensates when aggresomes are eliminated

using nocodazole. Lastly, we show that OptoTau-ΔN induces irreversible aggregates including granular tau oligomers in the cytoplasm, and confirm an increase in tau seeding activity. Collectively, our findings support a model whereby P301Ltau clustering is initially accumulated in aggresomes, and disruption of aggresomal function leads to tau aggregation through LLPS, ultimately forming in tau seeds.

The cellular mechanism of tau droplet formation and its role in neurofibrillary tangles formation.

Accumulation of tau in aggresomes

Numerous studies using human subjects, animals, and cultured cells have consistently shown the presence of hyperphosphorylated tau in cells during the emergence of tau pathology [8,73-75 76]. Our study's results, which show that oligomers of OptoTau are in a hyperphosphorylated state, support the previous reports. On microtubules, small tau oligomers such as dimers and trimers were detected ex vivo [77]. Phosphorylation of tau has been shown to promote tau axonal transport[78,79]. P301Ltau transgenic mice showed the ectopic expression of tau by somatodendritic mislocalization [80], and an increase in tau phosphorylation at S400 and S422 residues in the C-terminal region [23]. The previous reports and our findings give us the interpretation that oligomers of OptoTau may allow to move retrogradely transport on microtubules and reach the aggresomes.

Formation of aggresomes has been considered to occur compensatory when the capacity of the proteasome is exceeded by producing aggregation-prone misfolded proteins [46]. Inhibiting the proteasome is sufficient to drive the formation of tau-positive structures with the morphology of aggresomes [81,82]. The aggresome marker HDAC6 contributes to the formation of tau aggresomes [81]. These findings suggest that aggresomes provide an alternative pathway for the degradation and removal of harmful tau aggregates [83,84 85]. In contrast to previous studies, this study showed that tau oligomers were deposited on the aggresomes in the absence of proteasome inhibitors. The result suggests that degradation of tau on the aggresomes may occur even if the ubiquitin-proteasome system is not impaired at an early stage. The formation of tau clusters in tau-CRY2 experiments led to this new insight. Therefore, our results suggest that the early stages of tau oligomer formation might lead to the degradation of tau-CRY2 through aggresome

formation.

Transition of liquid phase to solid phase

Tau oligomers including dimer and trimer were detected on microtubules ex vivo [77]. Our findings also showed that OptoTau was clustered on microtubules and increased in tau phosphorylation by blue light. Zhang and colleagues using wild-type full-length tau construct fused to CRY2-mCherry showed that tau phase separation as condensate enhanced its binding to microtubules [86]. The proline-rich domain (a.a.151-245) in tau drives LLPS via the control of its phosphorylation state [86], supporting our findings. Pseudo-phosphorylated tau at S262 (S262E-OptoTau; figure S6), which strongly reduces the binding of tau to microtubules [43,87], was clustered in the cells by blue light irradiation, indicating that the condensate formation may not be attributed to the artificial effects of nocodazole. Pretangle, the most commonly observed neuronal lesion in Alzheimer's disease, is a poorly formed cytoplasmic inclusion, which is positive for phosphorylated tau [88]. The condensates of OptoTau in the cytoplasm were found to be reversible and not an insoluble aggregate like neurofibrillary tangles (NFTs). Therefore, they can be considered an intermediate form of tau aggregates[89]. Jiang and colleagues detected the forming tau oligomers by blue light irradiation in cultured cells expressing 1N4R-wild type tau with CRY2olig [90]. Mutant P301Ltau significantly enhances both tau droplet formation[29] and tau oligomer formation [91], compared with wild-type tau in vitro. Recent pathological analysis has shown that the cytoplasmic AT8-positive pretangle before forming NFTs, appears to be droplets [92]. Therefore, in the cytoplasm, microtubule-unbound full-length P301Ltau initially form droplets through LLPS, leading to the forming tau oligomers.

Multiple studies have demonstrated that the N-terminal region of tau serves to suppress aggregation [93 94]. Our findings indicate that clustering of the OptoTau-ΔN resulted in the formation of insoluble granular tau oligomers. It has been established that the microtubule-binding repeat domain of 4R-Tau (K18)[39] and truncated tau forms by caspase [95] and calpain I [55] exhibit a heightened or fast propensity for aggregation in comparison to full-length tau. Ca^{2+} overload and influx by neuronal hyperexcitability may induce tau cleavage [96 97]. In fact, AMPA and NMDA receptor stimulation increased local somatodendritic translation and hyperphosphorylation of tau protein [98]. Additionally, the

microtubule-binding repeat (MTBR) undergoes LLPS [28]. Cryo-EM studies have shown that tau sequences containing amino acid residues 297-391 of tau (referred to as dGAE) are the common core structure of fibrils in tauopathies [11-16]. Moreover, the dGAE sequence was found to be capable of inducing the formation of Alzheimer's-like fibrillar core structures in vitro [99]. RNA bound the C-terminal regions of tau at R406 and H407, and enhanced the integrity of tau aggregated [100]. Therefore, our results show that ΔN-P301Ltau forms insoluble aggregates that are coherent with previous studies.

Tau seeds play an important role in tau propagation [62,101-104]. We found that the tau oligomers observed in OptoTau-ΔN-expressing cells have tau seeding activity. Considering in combination with the fact that thioflavin T is responsive for β-sheet rich fibrils structured [20], N-terminal cleaved tau is suggested to be tau seeds to form fibrils. Prior to the formation of this seeded tau, P301Ltau oligomers accumulated along microtubules and in the aggresomes. Pathological analysis of human brains aged 1 to 100 years shows that the earliest change is AT8-positive pretangle in proximal axons of locus coeruleus preceding AD tangles [105]. The events observed in our cells may mimic such human tau pathology. Although the question of what seed tau is needs to be carefully addressed, dextran tau forms granular tau oligomers but not long fibrils (Figure S7), and the dextran formed tau aggregates enhanced the propagation of endogenous tau in C57BL/6J mice [106]. Aggregates of dGAE increased endogenous tau phosphorylation [107], and phosphorylated high-molecular-weight tau derived from Alzheimer's disease brain had tau seeding activity [62]. These facts suggest that the granular tau oligomers generated in this study have seed activity.

Novelty and useful for light-induced model of tau aggregation using CRY2olig.

Previous important studies [20,21] indicate that elucidating the initiation of the granular tau aggregation process is essential to better understand the pathogenesis of the disease[108]. In this study, we found that OptoTau-ΔN accumulates in the cytoplasm and forms granular tau oligomers. While previous research has shown that recombinant full-length P301Ltau can form granular tau oligomers in vitro[91], our study is the first to produce granular tau oligomers in cells by artificial manipulation. In addition, for the first time, we demonstrated the intracellular formation of granular tau oligomers with seeding activity. These imply that P301Ltau is converted from a liquid phase with soluble properties to a

solid phase with insoluble properties in cells acquiring seed activity. In the liquid phase, tau local concentration is induced by aggresome disruption and LLPS, which precedes aggregation. Thus, this study presents a novel mechanism for the aggregation pathway leading to the formation of intermediate aggregates with seed activity. These intracellular events may be potential targets for tau-related diseases to inhibit the formation of granular tau oligomers before forming NFTs. The circularity of tau condensates decreased along the blue light in OptoTau-ΔN, but this did not lead to tau fibril formation. We speculate that the lack of fibril formation is due to the low amount of granular tau oligomers in the cells. Because granular tau oligomers self-associate to form fibrils [20], tau fibrils may be formed when the amounts of granular tau oligomers reach the singularity point.

Limitations of the study and future directions

This study, using CRY2olig fusion tau protein, cannot exclude the possibility that effects of CRY2olig or blue light exposure affect other cellular processes. We observed aggregation of P301Ltau in Neuro2a cells, an immortalized cell line. However, the formation of tau aggregation may differ between the cell line and living organisms. Further analysis using primary neurons or transgenic mice expressing tau with CRY2olig could provide a more precise understanding of the biological mechanism of tau aggregation. Although tau pathology is observed in both neurons and glial cells, the aggregation of tau in glial cells has not been explored in this study. This CRY2olig system might be useful in investigating the mechanism of tau aggregation in glial cells, where the contribution of MTOCs in the formation of aggresomes is well-established [46]. Granular tau oligomers have been reported to be associated with cell death [23,109], but this study did not address cell death, as the immortalized Neuro2a cells used have an intrinsic proliferative capacity that could mask tau-induced cell death. The precise mechanism by which granular tau oligomers form in the cytoplasm remains unclear. Unlike in vitro studies with recombinant protein, cells are complex and contain many interacting molecules. Investigating the mechanism of tau aggregation in cells could lead to the identification of new targets for tau-related drug discovery.

Conclusion

In conclusion, our study proposes a new model of cellular tau aggregation that P301Ltau fused with CRY2olig forms soluble tau oligomers in the cytoplasm by LLPS. Subsequently, the N-terminal cleaved of the soluble oligomers forms insoluble granular tau oligomers, which have seeding activity. Further investigation into the mechanisms of this model may provide insights for the development of novel therapies for tauopathies.

Acknowledgments

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Author contributions

Y.S. conceived and designed the study and drafted this article. H.Y. constructed cDNA. R.K. critically supported drafting the article. Y.S, H.B., and A.T. critically discussed the design of the experiments and the article.

Declaration of interests

The authors declare no competing interests.

Figure 1

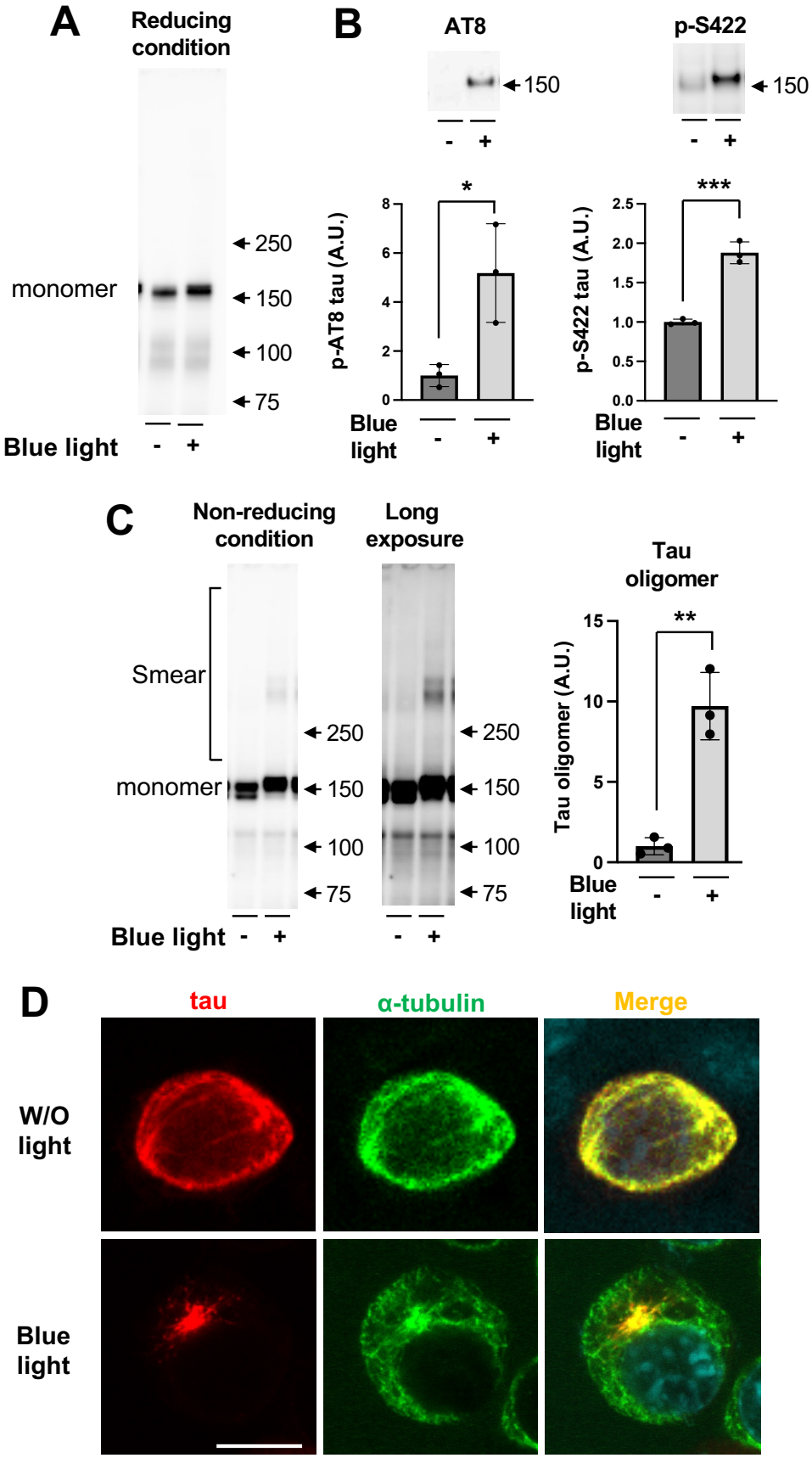
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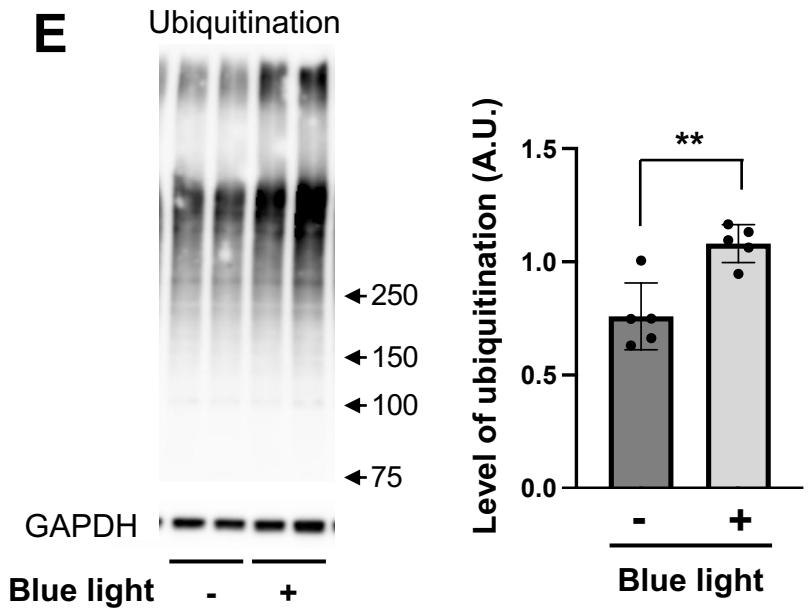
Figure 1 continued



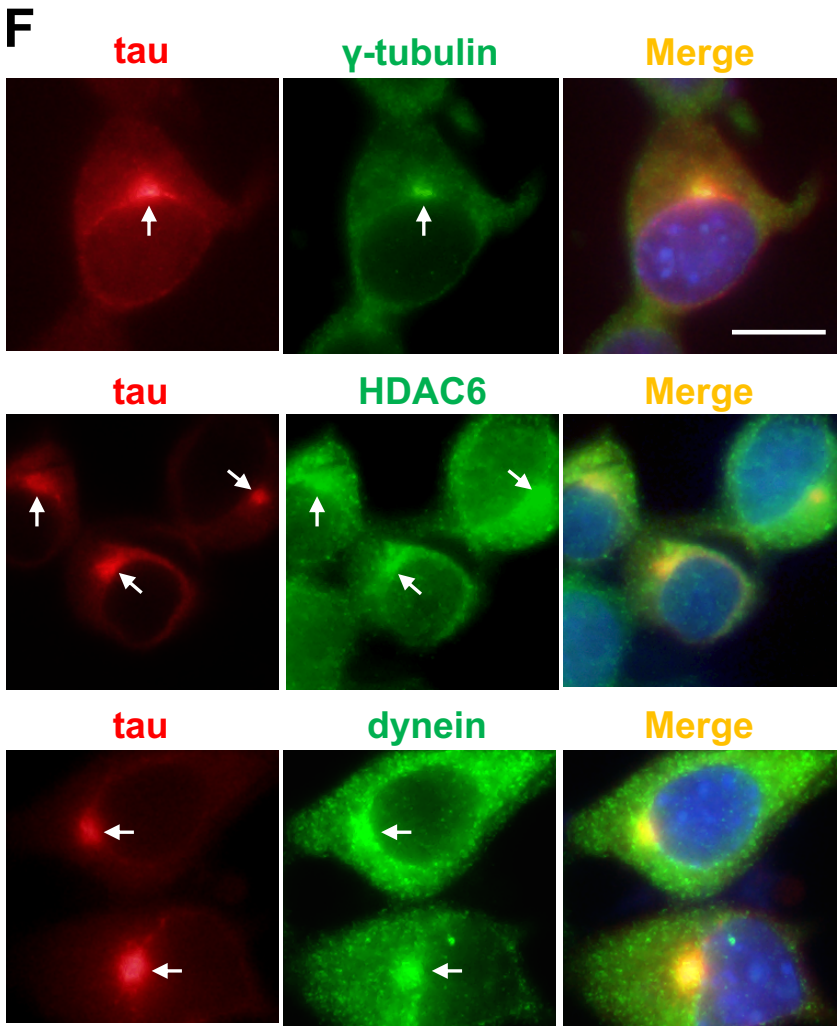


Figure 1: OptoTau is clustering in microtubules and transported to aggresomes.

(A,B) Under reducing condition, tau protein was detected by pan-tau JM antibody (A), and phospho-tau was detected by AT8 antibody which recognizes tau phosphorylation at S202 and T205 (B, left panel) and by p-S422-tau antibody (B, right panel) in Neuro2a cells expressing OptoTau with or without irradiation of blue light for 24 hours. Levels of phospho-tau were normalized to corresponding total tau (B, lower panel).

(C) Under non-reducing condition, the level of tau protein was determined by SDS-PAGE western blotting using JM antibody. To clearly detect the tau smear band on the membranes, a long period of exposure was employed (C, middle panel). The tau smear bands were quantified (C, right panel).

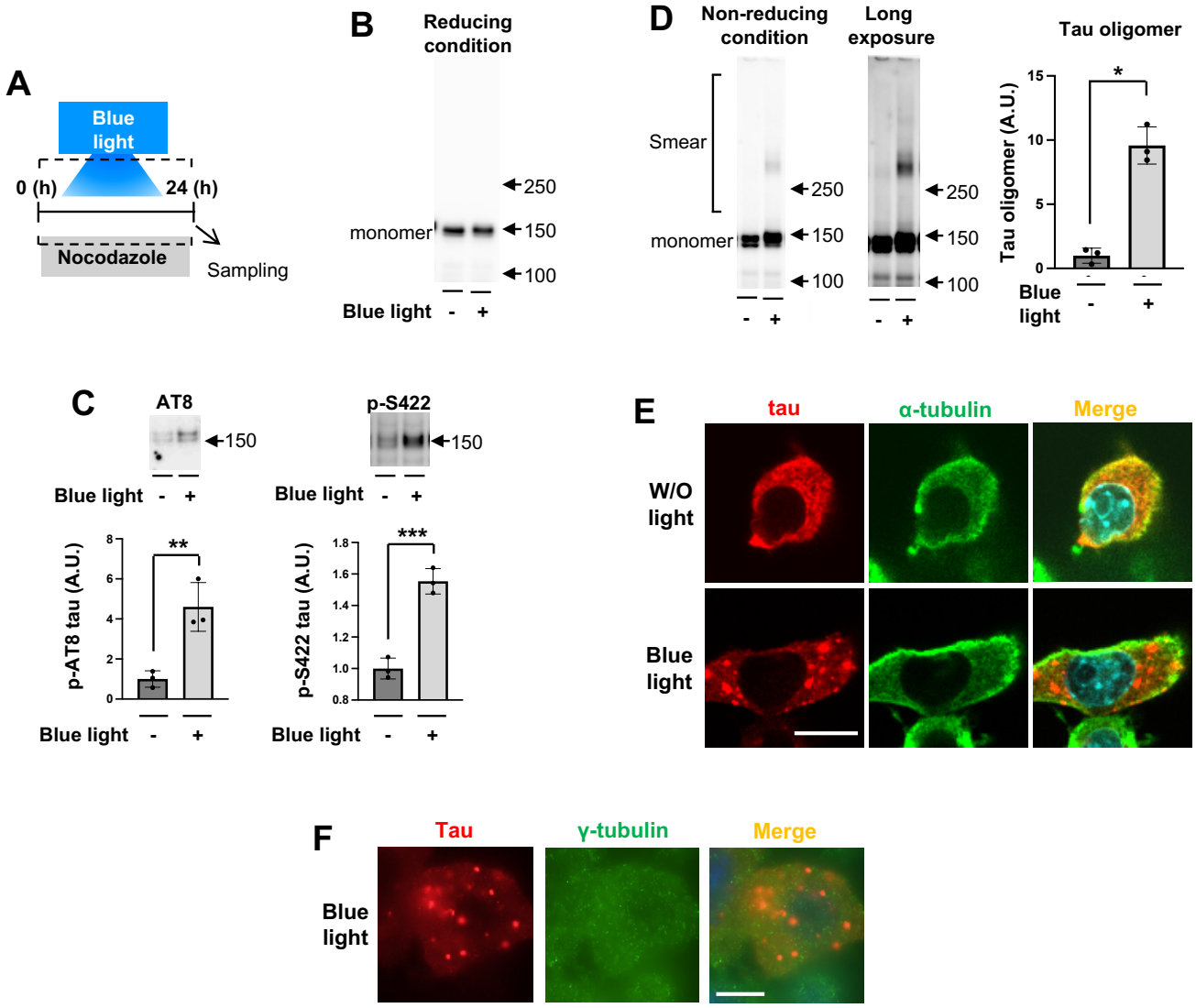
(D) Colocalization of OptoTau in microtubules. The cells were stained with SNAP-Cell TMR-Star to detect OptoTau as red fluorescence (D, left panel), and immuno-stained with α-tubulin antibody as green fluorescence (D, middle panel) 24 hours after blue light irradiation. Images of tau and α-tubulin were merged (D right panel).

(E) Ubiquitinated proteins were detected in Neuro2a cells expressing OptoTau with or without irradiation of blue light for 24 hours. The level of ubiquitinated proteins was normalized to corresponding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (E, right panel).

(F) Colocalization of OptoTau in aggresomes. After exposure to blue light for 20-24 hours, the cells were stained with SNAP-Cell TMR-Star (F, left panel, white arrow), and immuno-stained with γ-tubulin, histone deacetylase 6 (HDAC6) and dynein as green fluorescence (F, middle panel, white arrow). Images were merged (F, right panel).

All error bars indicate mean ± SD. *$p < 0.05$, ** < 0.01 and *** < 0.001 by Student's t-test. p-, phosphorylated. W/O, without. The white bar is 10 μm.

Figure 2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Nocodazole
Sampling
B
Reducing condition
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Tau
γ-tubulin
Merge
Blue light

Figure 2: OptoTau is concentrated in the cytosol by inhibition of microtubule assembly.

(A) Scheme of nocodazole exposure.

(B,C) Upon microtubules were depolymerized by nocodazole, tau protein was detected by pan-tau JM antibody (B), and phospho-tau was detected by AT8 antibody (C, left panel) and by p-S422-tau antibody (C, right panel) in Neuro2a cells expressing OptoTau with or without irradiation of blue light for 24 hours. Levels of phospho-tau were normalized by corresponding total tau (C, lower panel).

(D) Under non-reducing condition, the level of tau protein was determined by SDS-PAGE western blotting using JM antibody.

(E,F) The clustering of OptoTau under exposures to nocodazole and blue light for 24 hours was not colocalized with aggresomes. The cells were stained with SNAP-Cell TMR-Star to detect OptoTau as red fluorescence (E and F, left panel), and immuno-stained with α-tubulin antibody (E, middle panel) and γ-tubulin (F, middle panel) as green fluorescence. Images were merged (E and F, right panel).

All error bars indicate mean ± SD. *$p < 0.05$, ** < 0.01 and *** < 0.001 by Student's t-test. p-, phosphorylated. W/O, without. The white bar is 10 μm.

Figure 3

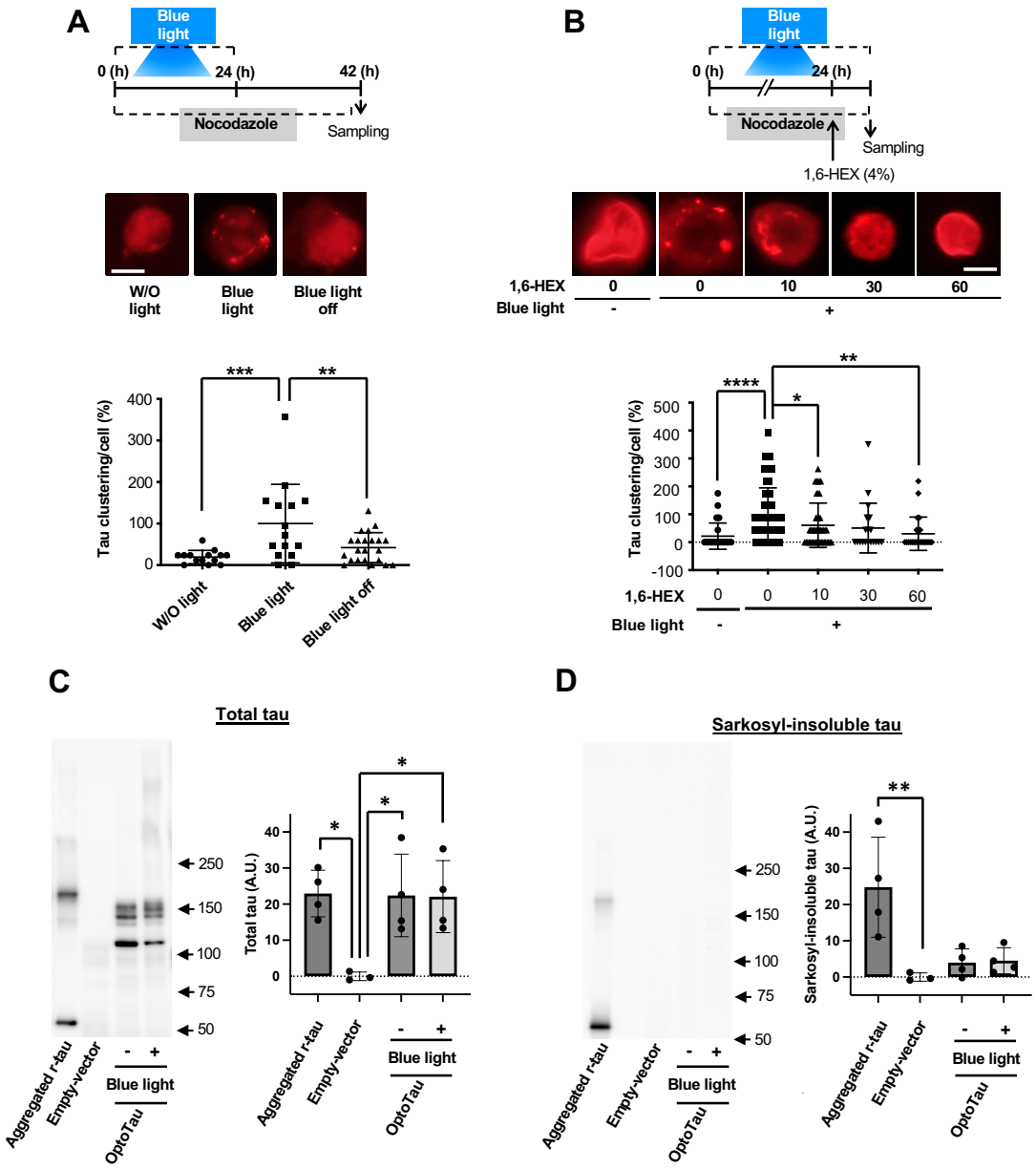


Figure 3: The reversible tau condensates in cells expressing OptoTau.

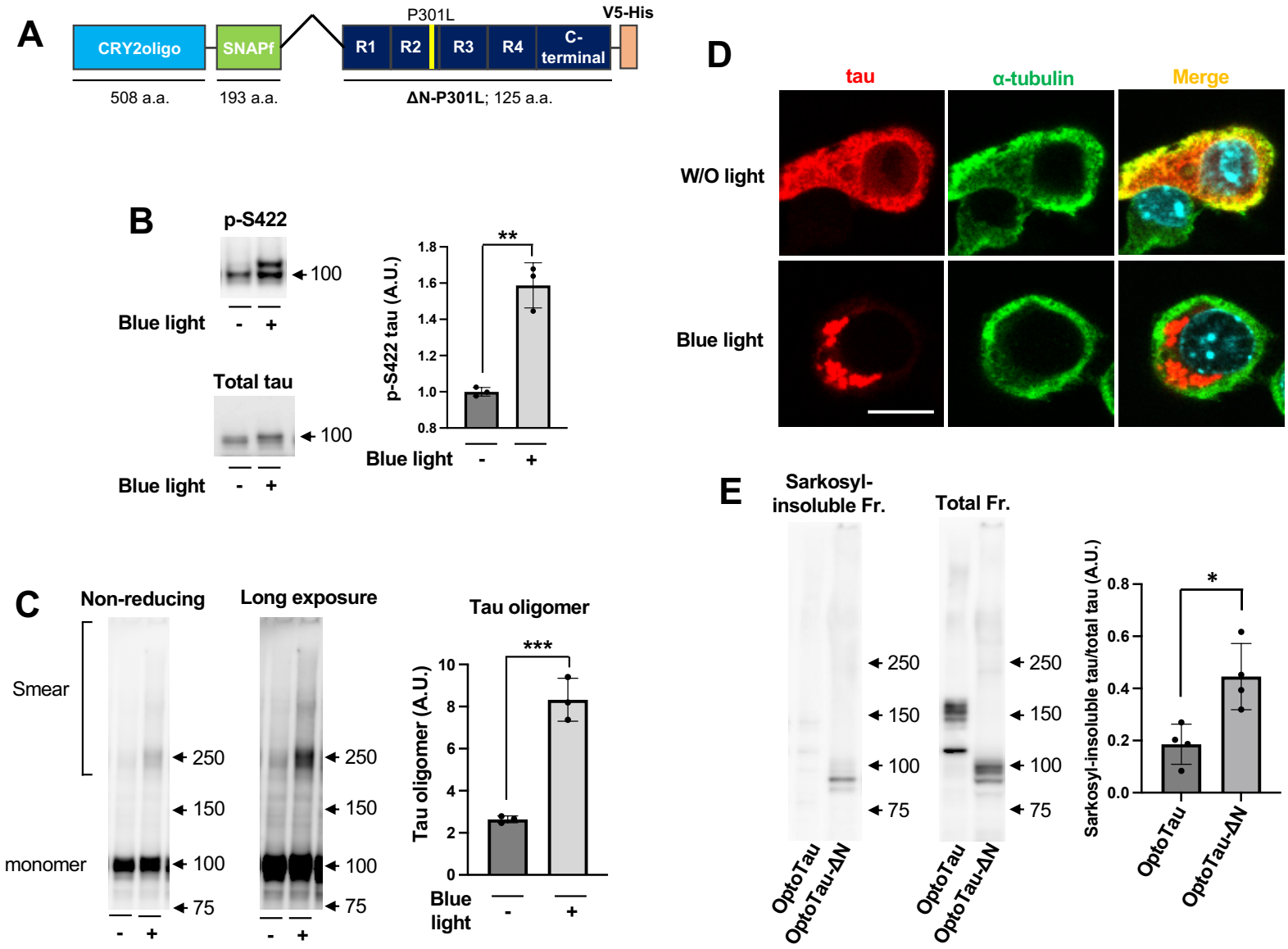
(A) Blue light was turned off after the cells expressing OptoTau under exposure of nocodazole were irradiated for 24 hours. The cells were stained with SNAP-Cell TMR-Star 18 hours after the light was turned off.

(B) 1,6-hexanediol (1,6-HEX) was added to the cells expressing OptoTau under exposures of nocodazole and irradiation for 24 hours.

(C-D) Clustering OptoTau under exposures to nocodazole and blue light for 23 hours was solubilized with anionic surfactant sarkosyl. After homogenates from cells expressing

OptoTau and empty vector, and aggregated recombinant tau were incubated with 1% sarkosyl for 1 hour at 37 ℃, and centrifuged. The levels of tau protein in the total fraction (C) and sarkosyl-insoluble fraction (D) were determined by SDS-PAGE western blotting using JM antibody (left panel). The tau bands were quantified (right panel).
All error bars indicate mean ± SD. *$p < 0.05$, ** < 0.01, *** < 0.001 and **** < 0.0001 by one-way analysis of variance and Dunnett's multiple comparisons test. The white bar is 10 μm. W/O, without. r-, recombinant.

Figure 4


A
CRY2oligo
SNAPf
P301L
R1
R2
R3
R4
C-terminal
V5-His
508 a.a.
193 a.a.
ΔN-P301L; 125 a.a.
B
p-S422
100
Blue light
- +
Total tau
100
Blue light
- +
p-S422 tau (A.U.)
1.8
1.6
1.4
1.2
1.0
0.8
**
Blue light
- +
C
Non-reducing
Long exposure
Smear
monomer
250
150
100
75
- +
Tau oligomer
Tau oligomer (A.U.)
10
8
6
4
2
0
***
Blue light
- +
D
tau
α-tubulin
Merge
W/O light
Blue light
E
Sarkosyl-insoluble Fr.
Total Fr.
250
150
100
75
OptoTau
OptoTau-ΔN
Sarkosyl-insoluble tau/total tau (A.U.)
0.8
0.6
0.4
0.2
0.0
*
OptoTau
OptoTau-ΔN

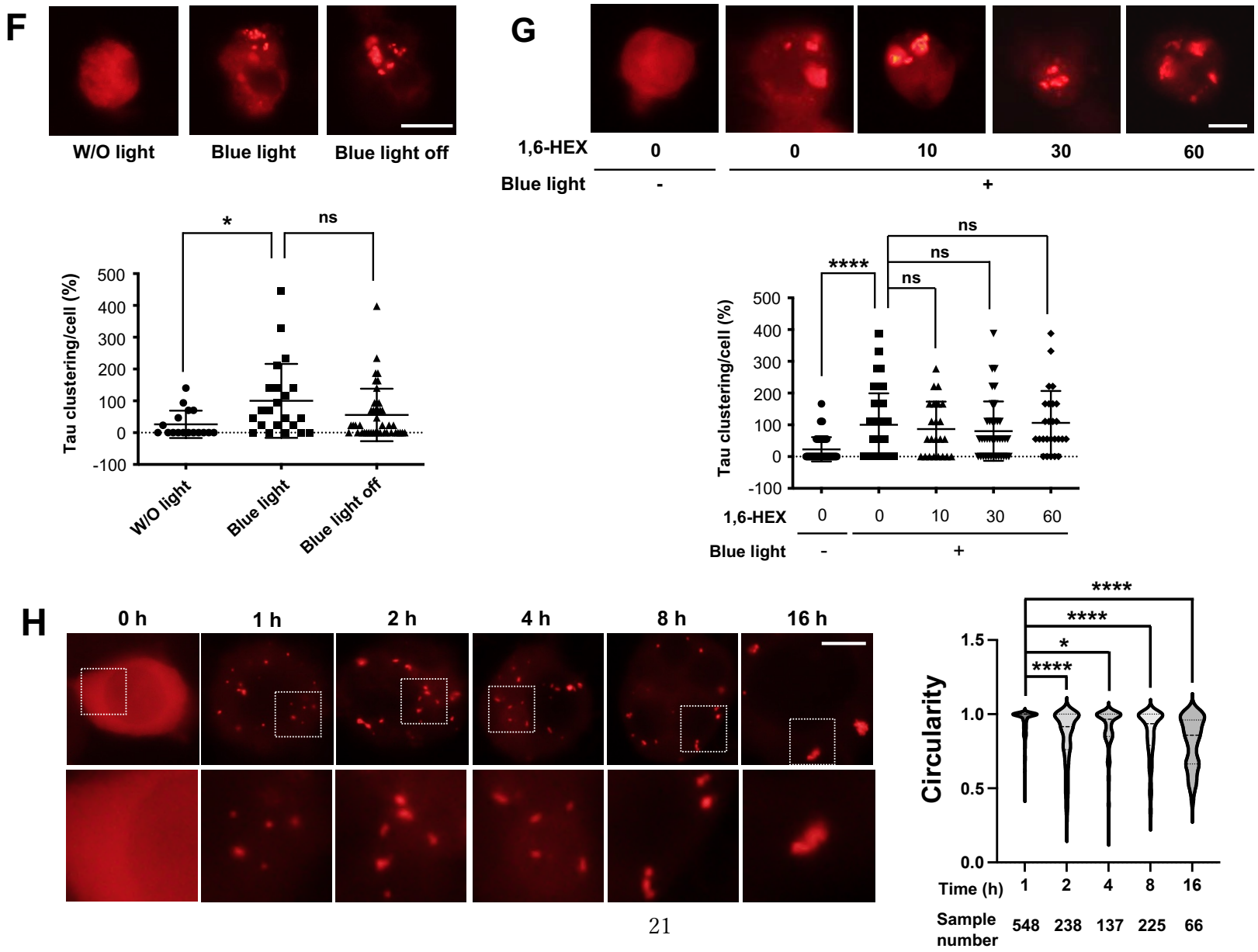

F
W/O light
Blue light
Blue light off
Tau clustering/cell (%)
500
400
300
200
100
0
-100
*
ns
W/O light
Blue light
Blue light off
G
1,6-HEX
0
0
10
30
60
Blue light
-
+
Tau clustering/cell (%)
****
ns
ns
ns
H
0 h
1 h
2 h
4 h
8 h
16 h
Circularity
1.5
1.0
0.5
0.0
****
*
****
****
Time (h)
1
2
4
8
16
Sample number
548
238
137
225
66

Figure 4 continued

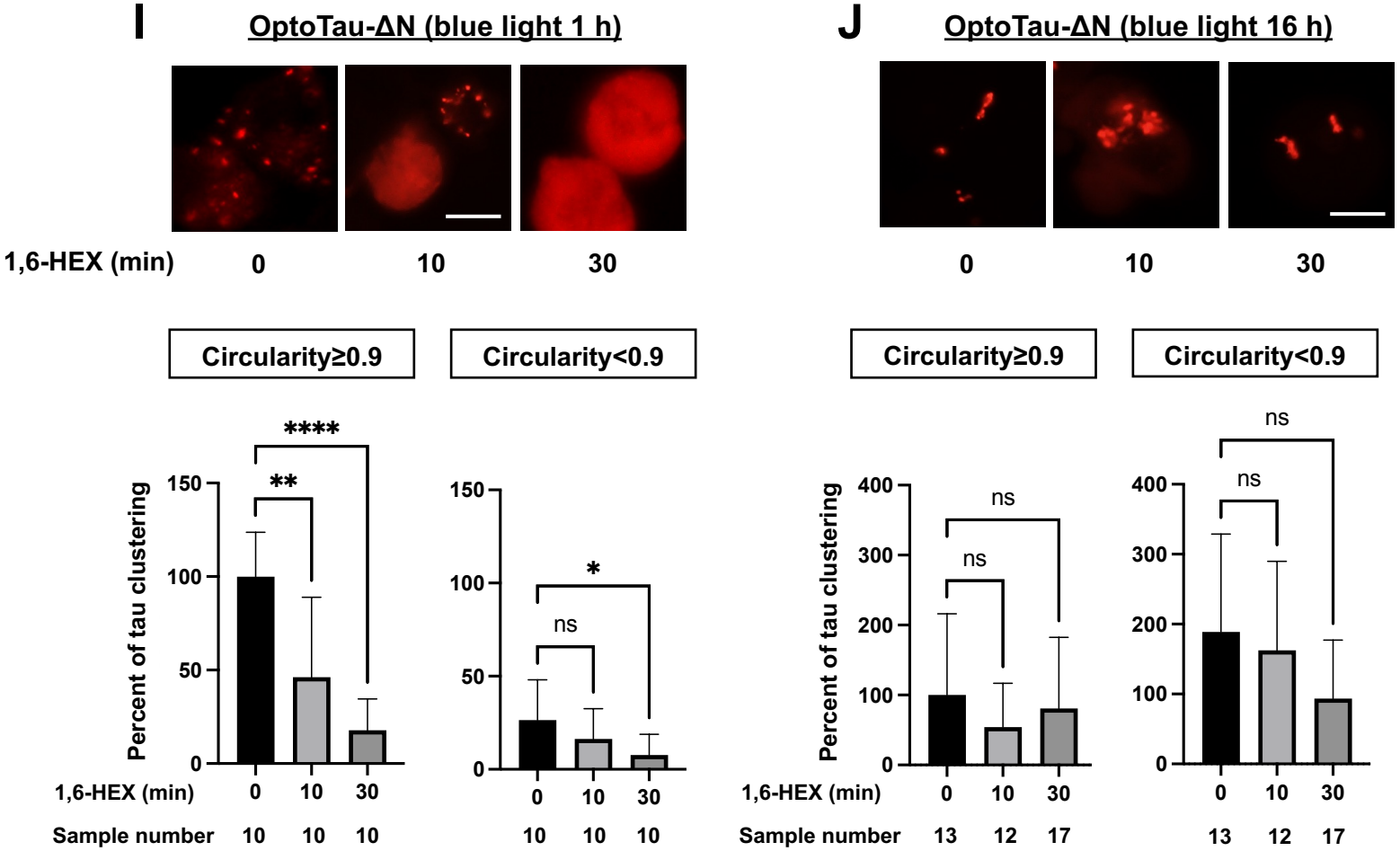


Figure 4: The irreversible tau condensates in cells expressing OptoTau-ΔN.

(A) Structure of OptoTau-ΔN.

(B) OptoTau-ΔN-expressing Neuro2a cells were exposed with nocodazole and blue light for 24 hours, then phospho-tau was detected by p-S422-tau antibody (left and upper panel) and tau protein was detected by pan-tau JM antibody (left and lower panel) using reducing condition SDS-PAGE western blotting. The level of phospho-tau was normalized to corresponding total tau (right panel).

(C) Under non-reducing condition, level of tau protein was determined by SDS-PAGE western blotting using JM antibody.

(D) The cells were stained with SNAP-Cell TMR-Star to detect OptoTau-ΔN as red fluorescence (left panel), and immuno-stained with α-tubulin antibody as green fluorescence (middle panel) 24 hours after exposures to nocodazole and blue light. Images were merged (right panel).

(E) OptoTau-ΔN was insolubilized with sarkosyl than OptoTau. Cells were collected and homogenized after exposures to nocodazole and blue light for 23 hours. The cell homogenates were incubated with 1% sarkosyl for 1 hour at 37 ℃. The levels of tau protein in the sarkosyl-insoluble fractions (left panel) and the cell homogenates (total fr., middle panel) were determined by SDS-PAGE western blotting using JM antibody. The tau bands were quantified, and levels of sarkosyl-insoluble tau were normalized by corresponding total tau (right panel).

(F,G) The clustering of OptoTau-ΔN under exposures to nocodazole and blue light grows into an irreversible aggregated structure. After the exposure for 24 h, the light was turned off for 18 hours (F) or the cells were treated with 1,6-hexanediol (1,6-HEX) (G). OptoTau-ΔN in the cells was stained with SNAP-Cell TMR-Star as red fluorescence.

(H) The circularity of tau clustering was time-dependently reduced by blue light. The cells expressing OptoTau-ΔN were exposed to blue light and 10 μM nocodazole at the indicated time, and stained with SNAP-Cell TMR-Star. The lower panels show expanded images in the dotted white square.

(I,J) The structure formed by the blue light short time exposure is a droplet formed by LLPS. The cells expressing OptoTau-ΔN were exposed with nocodazole and blue light for 1 (I) or 16 (J) hours, followed by incubation with 1,6-HEX for 0-30 minutes. The tau

clustering was stained with SNAP-Cell TMR-Star. The distribution of circular structures with a threshold of 0.9 and above (left panels) and non-circular structures with a threshold below 0.9 (right panels) was quantified using ImageJ. Tau clustering with circularity≥0.9 under no-treatment 1,6-HEX is expressed as 100%.

All error bars indicate mean ± SD. *$p < 0.05$, ** < 0.01, *** < 0.001 and **** < 0.0001 by Student's t-test (B,E), Welch's t-test (C) or one-way analysis of variance and Dunnett's multiple comparisons test (F,G,H,I,J). p-, phosphorylated. W/O, without. Fr, fraction. The white bar is 10 μm.

Figure 5

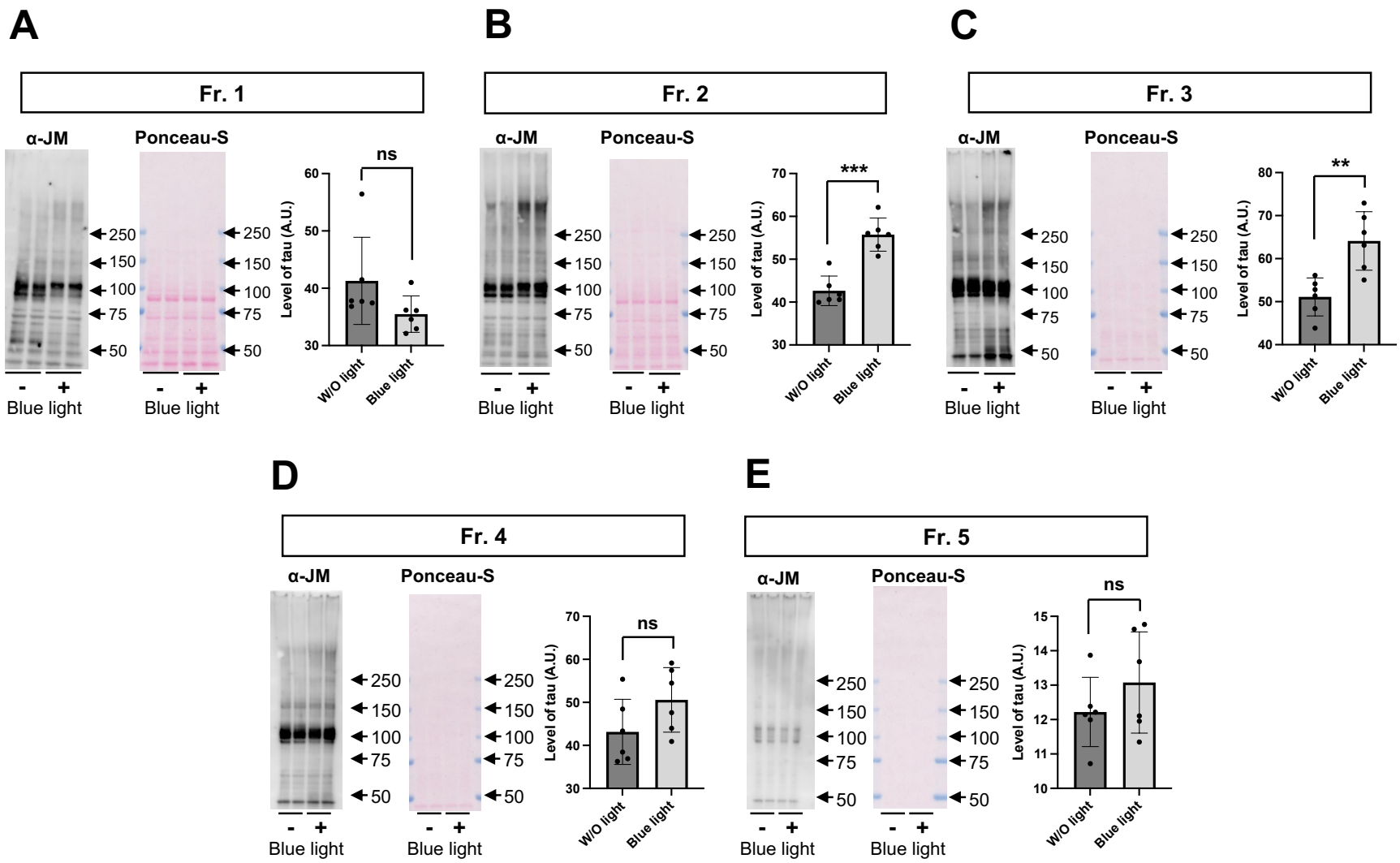

A
Fr. 1
α-JM
Ponceau-S
250
150
100
75
50
- +
Blue light
Level of tau (A.U.)
ns
W/O light
Blue light
B
Fr. 2
***
C
Fr. 3
**
D
Fr. 4
E
Fr. 5

Figure 5 continued

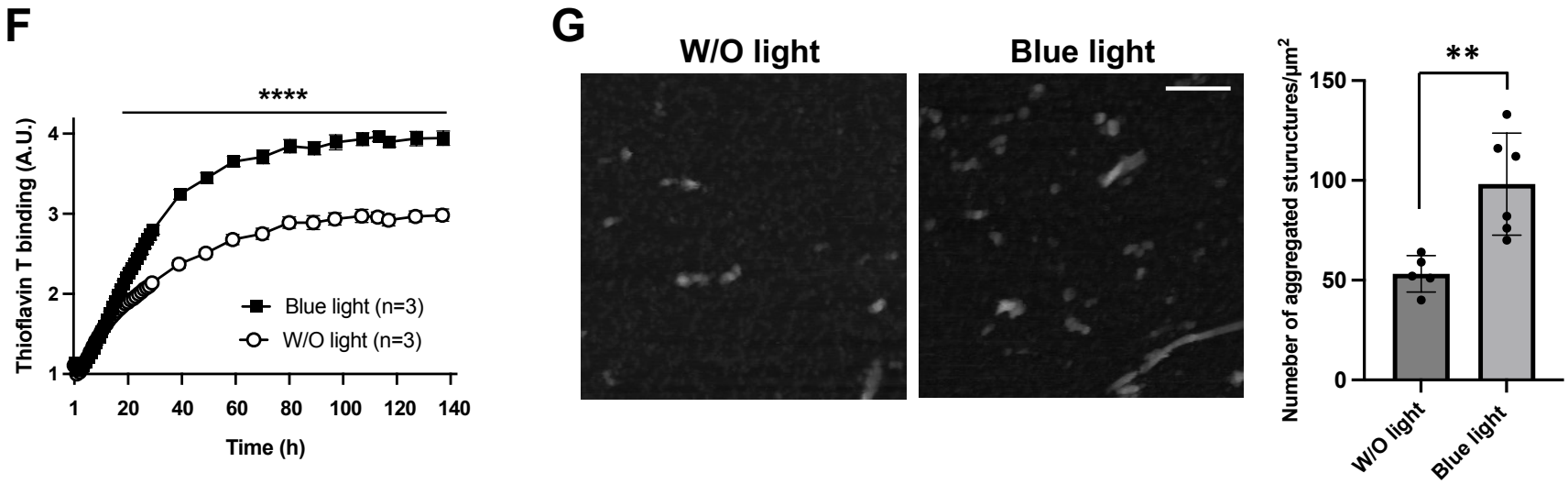


Figure 5: Characterization of solid-like state tau in cells expressing OptoTau-ΔN.

(A-E) The cells expressing OptoTau-ΔN were exposed to 10 μM nocodazole and blue light for 22 hours, and were lysed by 1% Triton X-100. After the lysates were separated into 5 fractions by sucrose density gradient centrifugation, tau protein and total proteins in fractions 1(A), 2(B), 3(C), 4(D) and 5(E) were detected with pan-tau JM antibody (right panel) and ponceau-S staining (middle panel), respectively. Levels of tau were normalized to corresponding ponceau-S staining (lower panel).

(F-G) Tau seeding activity was increased in cell lysates from blue light-exposed OptoTau-ΔN than W/O light. Recombinant tau protein was reacted with cell lysates and then polymerized by heparin. Recombinant tau aggregation was monitored by Thioflavin T binding at indicated times (Thioflavin T binding, $F(1, 168) = 5636$, $P<0.0001$; Time, $F(41, 168) = 1896$, $P<0.0001$; Interaction, $F(41, 168) = 107.9$, $P<0.0001$) (F). AFM detected tau aggregated structure in samples 140 hours after polymerization reaction (G, left and middle panels). The number of aggregated structures in the AFM images was counted and quantified (G, right panel).

All error bars indicate mean ± SD. ** < 0.01, *** < 0.001 and **** < 0.0001 by Student's t-test (A-E,G) or two-way ANOVA and Šídák's multiple comparisons test (F). W/O, without.

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STAR METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-pan-Tau (JM)	(Takashima et al., 1998)[110]	N/A
Mouse anti-tau antibody (tau5)	Thermo Fisher Scientific	AHB0042
Mouse anti-phospho-tau antibody (AT8)	Innogenetics	90206
Rabbit anti-phospho-tau antibody (p-S422)	Thermo Fisher Scientific	44-764G
Mouse anti-α-tubulin antibody	Sigma Aldrich-Merck	T-9026
Mouse anti-HDAC6 antibody	Santa Cruz	sc-28386
Mouse anti-γ-tubulin antibody	Santa Cruz	sc-17787
Mouse anti-dynein antibody	Santa Cruz	sc-13524
Mouse anti-GAPDH antibody	Millipore-Merck	MAB374
Horseradish peroxidase donkey anti-rabbit IgG (H+L)	Jackson Immuno Research	711-035-152
Horseradish peroxidase donkey anti-mouse IgG (H+L)	Jackson Immuno Research	715-035-151
Alexa Fluor™ 488 goat anti-rabbit IgG (H+L)	Thermo Fisher Scientific	A11008
Alexa Fluor™ 488 goat anti-mouse IgG (H+L)	Thermo Fisher Scientific	A32723
Alexa Fluor™ 555 goat anti-rabbit IgG (H+L)	Abcam	Ab150078
Alexa Fluor™ 568 goat anti-rabbit IgG (H+L)	Thermo Fisher Scientific	A11011
Alexa Fluor™ 568 goat anti-mouse IgG (H+L)	Thermo Fisher Scientific	A11004
Bacterial strain		
DH5α *E.coli*	TOYOBO	DNA-903F
BL21(DE3)	Biodynamics Laboratory Inc.	DS250
Chemicals, peptides, and recombinant proteins		
Triton X-100	FUJIFILM Wako Pure Chemical Corporation	160-24751
Tween-20	FUJIFILM Wako Pure Chemical Corporation	166-21115
SNAP-Cell TMR-Star	New England Biolabs	S9105S
SlowFade™ Gold Antifade Mountant with DAPI	Thermo Fisher Scientific	S36939

Methanol	Nacalai Tesque Inc.	21914
1,6-hexanediol	FUJIFILM Wako Pure Chemical Corporation	087-00432
Poly-D-lysine	Sigma Aldrich-Merck	P7886
Chemi-Lumi One L	Nacalai Tesque Inc.	07880
Chemi-Lumi One Super	Nacalai Tesque Inc.	02230
Thioflavin T	Sigma Aldrich-Merck	T3516
Heparin	Acros Organics	411210010
Sodium chloride	Nacalai Tesque Inc.	31320-05
Hepes	Dojindo	345-06681
4%-paraformaldehyde phosphate buffer solution	Nacalai Tesque Inc.	09154-56
Fetal bovine serum	biowest	S1810-500
Penicillin-streptomycin	Nacalai Tesque Inc.	26253-84
Trypsin solution	Nacalai Tesque Inc.	32777-15
Lipofectamine™ 2000	Thermo Fisher Scientific	52758
Nocodazole	Sigma Aldrich-Merck	M1404
Sucrose	FUJIFILM Wako Pure Chemical Corporation	196-00015
EDTA	Dojindo	340-01511
Pepstatin A	Nacalai Tesque Inc.	26436-52
Leupeptin	Nacalai Tesque Inc.	103476-89-7
Aprotinin	Nacalai Tesque Inc.	03346-84
Okadaic acid	Merck	459618-50UG
Sodium orthovanadate(V)	FUJIFILM Wako Pure Chemical Corporation	198-09752
Sodium fluoride	Nacalai Tesque Inc.	31420-82
β-Glycerophosphate disodium salt	FUJIFILM Wako Pure Chemical Corporation	048-34332
Protein assay CBB solution	Nacalai Tesque Inc.	11617-71
Protein assay BCA solution	Nacalai Tesque Inc.	06385-00
3-Mercapto-1,2-propanediol	FUJIFILM Wako Pure Chemical Corporation	207-09232
Sodium dodecyl sulfate (SDS)	Nacalai Tesque Inc.	02873-75
Glycerol	Nacalai Tesque Inc.	17018-25
Tris	Nacalai Tesque Inc.	02435-31

Bovine-serum albumin (BSA)	Sigma Aldrich-Merck	A3059
Ponceau-S	Sigma Aldrich-Merck	P3504
4x Laemmli SDS sample buffer	Bio-rad	1610747
Cellufine phosphate	JNC Corporation	676943324
Quick-CBB for coomassie brilliant blue staining	FUJIFILM Wako Pure Chemical Corporation	299-50101
Immersion oil	Olympus	IMMOIL-F30CC
PrimeSTAR MAX DNA Polymerase	Takara Bio Inc.	R045
Oligonucleotides		
pcDNA4V5His-KpnI-CRY2olig-F CTTAAGCTTGGTACCATGAAGATGGACA AAAAGACTATAG	This paper	N/A
CRY2olig-GSx2-R GGATCCCCCTCCTCCGGACCCTCCGCCT CCCGCTGCTCCGATCATGATC	This paper	N/A
GS-SNAPf-F GGAGGAGGGGGATCCGACAAAGACTGC GAAATGAAGC	This paper	N/A
SNAPf-GSx2-R GAATTCGGACCCTCCTCCTCCGGACCCG CCTCCGCCACCCAGCCCAGGCTTGCC	This paper	N/A
GS-tauNterm-F GGAGGAGGAGGGTCCgaattcGCTGAGCCC CGCCAGGAG	This paper	N/A
tauCterm-woSTOP-pcDNA-R GCCCTCTAGACTCGAGATCAAACCCTGC TTGGCCAG	This paper	N/A
GS-MTBR1-F GGCGGCTCCGAATTCGCCCCCGTGCCCA TGCCAG	This paper	N/A
Recombinant DNA		
Human full-length wild-type tau in pRK172	(Maeda et al., 2007) [20]	N/A
Human full-length P301L tau-CRY2olig (OptoTau) in pcDNA4 V5-His(B)	This paper	N/A
Human ΔN-P301L tau-CRY2olig (OptoTau-	This paper	N/A

ΔN) in pcDNA4 V5-His(B)		
P301L-2N4R tau constructed in PCI-neo	This paper	N/A
pSNAPf-ADRβ2 control plasmid	New England Biolabs	101123
pcDNA3.1 myc/His ver. B vector	Thermo Fisher Scientific	V855-20
Software		
ImageJ	National Institute of Health	https://imagej.nih.gov/ij/
Prism 9	GraphPad	https://www.graphpad.com
cellSens Standard 3.2	Olympus	https://www.olympus-lifescience.com/
Other		
Cell culture dish (10 cm)	CORNING	353003
6-well multiple plate	CORNING	3506
12-well multiple plate	CORNING	3513
Cover glass (Thickness 0.13-0.17 mm)	Matsunami Glass Ind.,Ltd.	N/A
Blue light irradiation device	CCS Inc.	TH2-211X200BL PD3-10024-8-PI
Cell scraper S	Sumitomo Bakelite Co., Ltd.	MS-93100
Polytron homogenizer	NITI-ON	NS-310E
Olympus BX50 microscopy	Olympus	N/A
FV3000 confocal laser scanning microscopy	Olympus	N/A
DP74 microscope digital camera	Olympus	N/A
Objective lens x100	Olympus	UPlanApo 100x/1,35 Oil Iris
Objective lens x40	Olympus	UPlanApo 40x/0,85
NuPAGE™ 3 to 8%, Tris-Acetate	Thermo Fisher Scientific	EA03755BOX
SuperSep™ Ace 5–20% gel	FUJIFILM Wako Pure Chemical Corporation	194-15021
Nitrocellulose membrane	Cytiva	10600001
NAP10 column	Cytiva	17-0854-02
Freeze dryer	EYELA	FDS-1000
TLA-110 fixed angle rotor	Beckman Coulter	366735
TLS55 swinging rotor	Beckman Coulter	346936
1.5 mL polypropylene tube	Beckman Coulter	357448

Open-top thinwall ultra-clear tube	Beckman Coulter	347356
Semi-dry transfer systems	Bio-rad	1703940
Amersham™ Imager 600	cytiva	N/A
NAP™-25	cytiva	17-0852-02
COSMOSIL(R) Protein-R Packed Column	Nacalai Tesque Inc.	06532-31
96-well plate optical black bottom plate	greiner bio-one	675096
Plate sealing tape	Sumitomo Bakelite Co., Ltd.	MS-30020
BioTek Synergy HTX muti-mode reader	Agilent	N/A
miVac centrifugal concentrators	Scrum Inc.	miVac DNA
Ultrasonic dispersion	SMT Co., Ltd.	UH-50
Atomic force microscopy (AFM)	Shimadzu Corp	SPM9700
AFM cantilever	Olympus	BL-AC40TS-C2

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yoshiyuki Soeda (soeday7188@gmail.com or yoshiyuki.soeda@gakushuin.ac.jp).

Materials availability

All unique/stable reagents generated in this study are available from the lead contact with a completed materials transfer agreement.

Data and code availability

・All data reported in this paper will be shared by the lead contact upon request.

・This paper reports no original code.

・Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell line

The mouse neuroblastoma cell line, Neuro2a, was cultured in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1x penicillin-

streptomycin. The culture was maintained in a 10 cm dish at 37 °C under 5% CO2.

METHOD DETAILS

cDNA construction

The cDNAs of tau and SNAPf were amplified using PrimeSTAR MAX DNA Polymerase from P301L-2N4R tau constructed in PCI-neo and pSNAPf-ADRβ2 control plasmid, respectively. The CRY2olig cDNA was prepared through site-directed mutagenesis (E409G) of CRY2 gene that was obtained from Addgene. The PCR products were subcloned into pcDNA3.1 myc/His ver. B vector. All primers used for this study are listed in the KEY RESOURCES TABLE. The cDNAs were transformed into DH5α, and their glycerol stocks were stored at -80 °C.

Transfection

Neuro2a cells at about 90% confluency were detached using 0.25 g/L trypsin and counted. For biochemical analysis, cells at 2.0 x 10^5 cells /ml were plated in a 6-well plate (2 ml) or 12-well plate (1 ml). For immunofluorescence analysis, cells at 1.0 x 10^5 cells /ml were plated onto cover glass coated with poly-D-lysine. cDNAs (1 μg) were transfected by lipofectamine 2000 according to the instruction manual 1 day after culturing. The transfection reagents were removed by a medium change 6-8 hours after transfection.

Irradiation of blue light

The transfected cells were exposed without or with 10 μM nocodazole in serum-free DMEM 1 day after transfection. The cells were placed onto a transparent plastic case with a height of 3 cm [111]. After setting the scale of the blue light irradiation device to 029, the cells were irradiated with blue light at 0.87 ± 0.16 mW/cm^2 for 1-24 hours. To serve as a control, aluminum foil was utilized to enclose the cell plates and prevent exposure to ambient light.

Preparation of cell homogenate

The cells in the medium were scraped, and then cell samples including the medium were centrifuged (15000 rpm, 2 minutes, 4°C). After removing the supernatants, the pellets

were homogenized in a buffer (250 mM sucrose, 1 mM EDTA, 10 mM Hepes [pH=7.4]) containing protease inhibitors (5 μg/ml pepstatin A, 5 μg/ml, leupeptin, 2 μg/ml, aprotinin), and phosphatase inhibitors (1 μM okadaic acid, 1 mM sodium orthovanadate(V), 1 mM sodium fluoride, and 1 mM β-glycerophosphate disodium salt) using a polytron homogenizer for 30 seconds at a setting to 10. After the homogenized solutions were centrifuged (3000xg, 10 minutes, 4°C), supernatants were recovered[112]. The protein concentration of the samples was measured with the Bradford assay. To analyze under reducing condition, samples were suspended in 2.5% 3-mercapto-1,2-propanediol-containing 1x Laemmli SDS sample buffer followed by boiling for 10 minutes. Under non-reducing condition, the samples were suspended in sample buffer containing 0.5% SDS, 10% glycerol, 0.002% bromophenol blue and 62.5 mM Tris-HCl (pH=6.8).

Staining of tau with CRY2olig

The tau cDNAs were fused to CRY2olig with a SNAPf tag (figure S1), which can covalently bind to chemical fluorescent dyes. SNAP-Cell TMR-Star (excitation wavelength, 554 nm; fluorescence wavelength, 580 nm) was used to label the tau protein with CRY2olig in cells. The cells on cover glass coated with poly-D-lysine were fixed with prewarmed 4% paraformaldehyde (PFA) for 15 minutes at room temperature. After washing with phosphate-buffered saline (PBS), the cells were treated with 0.1 μM SNAP-Cell TMR-Star and 1% bovine serum albumin (BSA) in PBS, and incubated at 4°C in a moist and dark box over-night.

Immunofluorescence

The cells were fixed with prewarmed 4% PFA for 15 minutes at room temperature to detect-α-tubulin and HDAC6. To detect γ-tubulin and dynein, the cells were fixed by methanol at -30°C for 15 minutes followed by 4% PFA at room temperature for 5 minutes. After permeabilization with 0.1% TritonX-100, the cells were blocked by 1% BSA containing 0.1% Tween 20 in PBS buffer. The cells were treated with primary antibodies (anti-α-tubulin antibody [1:500], anti-HDAC6 antibody [1:100], anti-γ-tubulin antibody [1:100], and anti-dynein antibody [1:100]) and SNAP-Cell TMR-Star (0.1 μM) in 1%

BSA containing 0.1% Tween 20 in PBS buffer and incubated at 4℃ over-night. After washing with PBS, incubation at room temperature for 1 hour was conducted in 2nd antibodies with Alexa fluor (1:1000) in 1% BSA containing 0.1% Tween 20 in PBS buffer.

Microscopy observation and image analysis

After staining, the cells were mounted using SlowFade™ gold with DAPI and observed under reflected light microscopy (figures 1F, 2F, 3A, 3B, 4F, 4G, 4H, 4I, and 4J) and confocal microscopy (figures 1D, 2E, and 4D) with oil immersion objective lenses x100 or x40. Images were obtained using a digital camera DP74 and image software, cellSens Standard 3.2. Tau clusters stained by SNAP-Cell TMR-Star were counted. To measure the circularity of tau clusters in cells, analyze particles in ImageJ was performed after images were adjusted for brightness/contrast and binarized.

Experiments of light off and 1,6-hexanediol exposure.

After cells expressing OptoTau and OptoTau-ΔN were irradiated with blue light for 24 hours, the cells were cultured in DMEM containing 10 μM nocodazole without FBS. For the light off experiment, the culture was performed for 18 hours without blue light irradiation. The cultures were carried out under aluminum foil to prevent the influence of ambient light. For the 1,6-hexanediol exposure experiment, cells were cultured in DMEM with 4% 1,6-hexanediol for 10-60 minutes at 37 ℃ in a CO_2 incubator. Tau proteins in these cells were stained with SNAP-Cell TMR-Star.

Preparation of sarkosyl-insoluble fraction

Cells in three wells of the 6-well plate were collected 24 hours after blue light irradiation. A negative control is cells expressing an empty vector. To prepare the positive control, recombinant tau (10 μM) was polymerized with heparin for 7 days, and diluted to 1 μM. Cells and positive control were homogenized in 300 μl of the homogenization buffer (see Preparation of cell homogenate), and centrifuged at 1000 x g for 10 minutes to remove nuclear. One hundred eighty μl of the supernatant (total fraction) was transferred into 1.5-ml tube, and mixed 20 μl of 10% TritonX-100 (final concentration, 1%). After rotating for 20 minutes at 4 ℃ to solubilize other organelles, the supernatants were centrifuged

(65,000 rpm, 1 hour, 4 °C) in a TLA110 swinging rotor. After removing the supernatant, 200 μl of 1% sarkosyl was added to the pellets. The pellets were sonicated for 1 second at a setting to 1. This operation was repeated until the pellet was invisible to the human eye. After rotating for 1 hour at 37 °C, centrifugation (65,000 rpm, 1 hour, 4 °C) was conducted. Pellets were washed with milliQ water to remove trace soluble tau, and solubilized in 70% formic acid. After the formic acid in the samples was removed by evaporating, pellets were suspended in 30 μl of 1x Laemmli SDS sample buffer containing 2.5% 3-mercapto-1,2-propanediol and boiled. If the color of the sample was yellow, it was restored to blue by adding a small amount of 1 M Tris (pH=7.4).

Sucrose density gradient centrifugation

Sucrose density gradient centrifugation was performed as previously described [21] with minor modifications. Cell homogenates from OptoTau-ΔN-expressing cells were solubilized with 1% TritonX-100 at 4 ℃ for 20 minutes to solubilize cell organelles [113]. High-molecular-weight tau formed by irradiation of blue light was not solubilized by 1% Triton X-100 (figure S8). Sucrose step gradients (each containing 450 μl of 10, 20, 30, 40 and 50% sucrose in buffer including 10 mM hepes and 1 mM EDTA) were prepared in an open-top thinwall ultra-clear tube. The samples (450 μl) were layered on top of the sucrose step gradients, and centrifuged (50,000 rpm, 2 hours, 4 ℃) in a TLS55 swinging rotor and separated into 5 fractions. The recovered fractions were suspended in the non-reducing sample buffer and applied to SDS-PAGE western blotting.

SDS-PAGE western blotting

The samples from the preparation of cell homogenate and the sucrose density gradient centrifugation were separated by NuPAGE™ 3 to 8% tris-acetate gel or SuperSep™ Ace 5–20% gel. Proteins in the gels were transferred onto nitrocellulose membranes using semi-dry transfer systems. The membrane was blocked with 3% milk in PBS-T for 1 h at room temperature. They were probed with antibodies including JM (1:1000-1:2000), AT8 (1:750), p-S422 (1:1000) and GAPDH (1:5000) antibodies in 3% BSA for overnight at 4 °C. After washing the membrane with PBS-T, blots were incubated with horseradish peroxidase-linked secondary antibodies (1:5000) in PBS-T. The membranes were washed

and examined by enhanced chemiluminescence detection (Chemi-Lumi One L or Chemi-Lumi One Super) on an Amersham™ Imager 600. To quantify band intensity, ROIs in the image were defined by a rectangle, and the gray mean value was measured using ImageJ. Levels of phospho-tau were normalized to corresponding total tau detected by the JM antibody.

Preparation of recombinant tau protein

Human wild-type tau (2N4R) cDNA was cloned into a pRK172 vector[20]. Recombinant tau was expressed in *E. coli* BL21 (DE3) and purified by the modified method previously reported [20]. After sonication and boiling of the *E. coli*, recombinant tau protein in the heat-stable fraction was purified by ion-exchange chromatography (Cellufine Phosphate), ammonium sulfate fractionation, gel filtration chromatography (NAP10 column) and reverse phase-HPLC (COSMOSIL Protein-R Waters). After freeze-drying, recombinant tau proteins were dissolved in milliQ water and stored at -80 °C as a stock solution. The concentration of tau protein was determined relative to BSA as the standard by coomassie brilliant blue staining (Quick-CBB) after the separation of tau protein by SDS-PAGE.

Tau seeding activity

Cell homogenates were obtained from cells expressing OptoTau-ΔN with or without irradiation of blue light by the aforementioned preparation of cell homogenate. They were incubated in 1% TritonX-100 at 4 ℃ for 20 minutes to prepare cell lysates. The concentration of total proteins in the cell lysates was adjusted to 0.8 mg/ml. After mixing recombinant wild type-tau protein (10 μM) and Thioflavin T (10 μM) in Hepes buffer (10 mM Hepes, pH=7.4; 100 mM NaCl), 4 μl of the cell lysates were added. The samples were incubated at room temperature for 15 minutes, and then heparin (0.06 mg/ml) was added. Three reactions were prepared in a 96-well plate optical black bottom plate to a final total volume of 25 μl. The plate was covered with sealing tape and incubated at 37 °C in a plate reader. The kinetics of tau polymerization were monitored in real-time by measuring the fluorescence intensity every hour at 420/27 nm excitation and 500/27 nm emission from the bottom of the plate. Shaking was performed for 10 seconds of linear shaking at 550 rpm before the reading of fluorescence intensity.

Atomic force microscopy (AFM)

The AFM equipped with a dynamic mode was employed for the observations of recombinant tau aggregated structure [20,114]. After tau seeding activity, 2 µl of tau aggregation mixtures was diluted by 18 µl of milliQ-water and was deposited onto freshly cleaved mica. The mica was incubated for 10 minutes at room temperature in a moist box, and washed by 100 or more µl of milliQ-water. An AFM microcantilever scanned the mica at 0.3 Hz. The resolution of images is 512*512 pixels. After the Images were adjusted by threshold and binarized, the number of aggregated structures was counted by the analyze particles in ImageJ. The threshold for the analysis was set at 2.257 nm on the major axis, which is difficult to distinguish from noise.

Statistical analysis

All statistical tests were conducted using GraphPad Prism 9. The significance of differences between two groups was assessed by Student's or Welch's t-tests following an F-test. Differences between multiple groups were assessed by one-way analysis of variance and Dunnett's multiple comparisons test. Data from tau seeding activity were analyzed using a two-way ANOVA. A p-value less than 0.05 was considered statistically significant.