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FTD-associated mutations in Tau result in a combination of dominant and recessive phenotypes

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Abstract

Although mutations in the microtubules-associated protein Tau have long been connected with several neurodegenerative diseases, the underlying molecular mechanisms causing these tauopathies are still not fully understood. Studies in various models suggested that dominant gain-of-function effects underlie the pathogenicity of these mutants; however, there is also evidence that the loss of normal physiological functions of Tau plays a role in tauopathies. Previous studies on Tau in *Drosophila* involved expressing the human Tau protein in the background of the endogenous Tau gene in addition to inducing high expression levels. To study Tau pathology in more physiological conditions, we recently created *Drosophila* knock-in models that express either wildtype human Tau ($hTau^{WT}$) or disease-associated mutant hTau ($hTau^{V337M}$ and $hTau^{K369I}$) in place of the endogenous *Drosophila* Tau (dTau). Analyzing these flies as homozygotes, we could therefore detect recessive effects of the mutations while identifying dominant effects in heterozygotes. Using memory, locomotion and sleep assays, we found that homozygous mutant hTau flies showed deficits already when quite young whereas in heterozygous flies, disease phenotypes developed with aging. Homozygotes also revealed an increase in microtubule diameter, suggesting that changes in the cytoskeleton underlie the axonal degeneration we observed in these flies. In contrast, heterozygous mutant hTau flies showed abnormal axonal targeting and no detectable changes in microtubules. However, we previously showed that heterozygosity for $hTau^{V337M}$ interfered with synaptic homeostasis in central pacemaker neurons

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Appendix A. Supplementary data

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and we now show that heterozygous hTau^{K369I} flies have decreased levels of proteins involved in the release of synaptic vesicles. Taken together, our results demonstrate that both mutations induce a combination of dominant and recessive disease-related phenotypes that provide behavioral and molecular insights into the etiology of Tauopathies.

Keywords

Drosophila; Microtubules; Memory; Sleep; Axonal degeneration

1. Introduction

The microtubule-associated protein Tau has long been connected to Alzheimer's disease (AD) and a number of other neurodegenerative diseases collectively called Tauopathies (Lee and Leugers, 2012; Lee et al., 2001). The relevance of Tau in the pathogenesis of these diseases has been confirmed by the identification of several mutations in Tau that cause Frontotemporal Dementia with Parkinsonism linked to chromosome 17 (FTDP-17) (van Swieten and Spillantini, 2007), which is second only to AD as the most common cause of dementia in patients under 65 (Basiri et al., 2015). A longstanding question in the field is whether these mutations in Tau lead to a loss of its normal function, a novel dominant function, or a combination of both. The best-described function of Tau is its role in interacting with microtubules and a number of the disease-causing mutations are localized in the microtubule-binding domains and reduce the ability to promote microtubule polymerization, at least *in vitro* (Combs and Gamblin, 2012; LeBoeuf et al., 2008). Furthermore, phosphorylation of Tau decreases its microtubule-binding affinity (Barten et al., 2012; Li et al., 2007); thus disease-related Tau hyperphosphorylation, as occurs in AD, can also interfere with Tau's microtubule interacting function (Trojanowski and Lee, 2005). That Tau's microtubule-associated functions play a role in pathology is also suggested by findings that microtubule mass is reduced in AD patients (Cash et al., 2003). This supports the hypothesis that a loss of microtubule-associated functions results in pathology (Combs and Gamblin, 2012; LeBoeuf et al., 2008). A loss-of-function mechanism is also supported by the observation that the lack of endogenous Tau aggravates the histological and behavioral phenotypes in a FTDP-17 mouse model (Ando et al., 2011), and FTDP patients exhibit reduced Tau levels (Papegaey et al., 2016; Zhukareva et al., 2003; Zhukareva et al., 2001).

However, most disease-associated Tau mutations have dominant effects, although a few are recessive or only cause phenotypes as compound heterozygotes (Anfossi et al., 2011; Ghetti et al., 2015; Nicholl et al., 2003; Rossi and Tagliavini, 2015), suggesting a pathogenic gain-of-function mechanism. This is further supported by data that expression of disease-associated mutant forms of human Tau in animal models recapitulates the phenotypes of FTDP-17 in the presence of endogenous Tau (Dujardin et al., 2015). Although this supports dominant effects, a novel function seems not to be required for Tau to be pathogenic because even wildtype Tau can be toxic when overexpressed. This has been shown in mice and flies where expression of wildtype Tau can induce degenerative and behavioral phenotypes although they are in general less severe compared to the expression of mutant Tau forms

(Brion et al., 1999; Ishihara et al., 1999; Jackson et al., 2002; Khurana et al., 2006). Due to these different findings, it has been suggested that a combination of both gain-of-function effects and loss of normal physiological functions of Tau may play a role in tauopathies (Arendt et al., 2016; Rossi and Tagliavini, 2015; Sotiropoulos et al., 2017).

Besides binding and stabilizing microtubules, Tau has now been shown to also have a variety of other functions but whether disruptions in these functions contribute to pathology is still unclear. Wildtype Tau has been connected with the regulation of synaptic plasticity, neurogenesis, cell signaling, and functions in the nucleus (Arendt et al., 2016; Guo et al., 2017; Rossi and Tagliavini, 2015; Sotiropoulos et al., 2017). In agreement with these additional functions, Tau has been localized to somatodendritic compartments, mitochondria, the plasma membrane, and the nucleus, although its levels are generally lower in these compartments than in axons (Hanger et al., 2019; Ittner and Ittner, 2018; Li et al., 2016; Morris et al., 2011; Regan and Cho, 2019; Tashiro et al., 1997). Decreasing Tau expression has been shown to result in synaptic loss and synaptic plasticity is impaired in Tau deficient mice (Ahmed et al., 2014; Chen et al., 2019). In *Drosophila*, knocking down endogenous dTau also results in a reduction in the number of synaptic sites and this phenotype is worsening with age (Voelzmann and Okenve-Ramos, 2016). At the presynapse, Tau has been demonstrated to interact with several synaptic proteins, affecting the clustering of synaptic vesicles and neurotransmitter release (McInnes et al., 2018). That disruptions of Tau's synaptic functions could play a role in pathogenesis is supported by findings that Tau accumulates at synapses in patients and animal models of tauopathy and that this correlates with the onset of cognitive decline (DeKosky and Scheff, 1990; Yoshiyama et al., 2007).

To investigate recessive as well as dominant effects of mutations in Tau on behavior, anatomy, and molecular functions of this protein, we created several novel knock-in *Drosophila* models. While *Drosophila* has already been well established as a tauopathy model, the previous models were based on the GAL4/UAS system (Cowan et al., 2010; Khurana, 2008; Sivanantharajah et al., 2019; Sun and Chen, 2015; Wentzell and Kretzschmar, 2010), with human Tau (hTau) being expressed in the presence of endogenous fly Tau (dTau). Therefore, this prevents the detection of recessive phenotypes and in addition hTau is expressed ectopically and often at high expression levels. In contrast, in our models hTau is expressed under the control of the dTau promoter and in the absence of endogenous fly dTau in homozygous transgenic flies. In addition to flies expressing wildtype hTau, we generated flies carrying the $hTau^{K369I}$ or $hTau^{V337M}$ mutation. In humans, $hTau^{V337M}$ has been shown to cause FTDP-17 (Frontotemporal Dementia and Parkinsonism linked to chromosome 17) which is characterized by personality changes and cognitive impairment, in addition to motor symptoms similar to the ones occurring in Parkinson's disease (Poorkaj et al., 1998; Sumi et al., 1992). $hTau^{K369I1}$ was identified in patients with Pick's disease who show changes in behavior, cognitive deficits, problems using language, and physical weakness (Neumann et al., 2001). Here, we demonstrate that both mutations induce a combination of dominant and recessive disease-related phenotypes at the behavioral and molecular levels in our models.

2. Materials and methods

2.1. Drosophila stocks

The knock-in lines were created by replacing the dTau coding region with the cDNA for the human Tau 1N4R isoform using the CRISPR/Cas9 genomic editing system. A more detailed description is given in (Cassar et al., 2020). These flies were originally in the w^{1118} background but were backcrossed to CS to prevent phenotypes due to the *w* mutation. The UAS-mCD8-GFP (BDSC 5130) line was kindly provided by M. Logan (OHSU), *Pdf*-GAL4 (BDSC 6899, from the Giebultowicz laboratory), UAS-EB1-GFP (Rolls et al., 2007) by M. Rolls (PennState University), and UAS-GFP-tub (BDSC 7374) by D. Applewhite (Reed College, Portland). Flies were maintained on standard fly food under a 12:12 h light: dark cycle at 25 °C. Wildtype Canton S (CS) from M. Heisenberg (University Wurzburg) served as control.

2.2. Fast phototaxis

Fast phototaxis assays were conducted in the dark using the countercurrent apparatus described by (Benzer, 1967) and a single light source. A detailed description of the experimental conditions can be found in (Strauss and Heisenberg, 1993). Flies were collected and aged with fresh food vials provided every 6–7 days. Flies were then tested in groups of 10–15 flies. Five consecutive tests were performed in each experiment with a time allowance of 6 s to make a transition towards the light and into the next vial and a value determined for each fly based on which of the six vials it reached. The mean value was then determined for the group. Statistical analysis was done using GraphPad Prism (San Diego, CA, United States). Normal distribution was verified with the D'Agostino and Pearson omnibus test and significances identified with ANOVA one-way analysis of variance and Dunnett's post-tests.

2.3. Short-term working memory assay

Flies with their wings cut to one-third of their length under cold anesthesia (one day before testing) were analyzed in an LED arena that has been described in (Neuser et al., 2008). Individual flies were tested 10 times for positive choices, *i.e.*, re-orientation towards the position of the initial, then vanished landmark after a detour to a shortly-presented distractor landmark at the side. When using homozygous hTau flies, we noted that many of the 3d old ones did not approach the initial landmark in a straight line but where sometimes hyperactive and "meandering" (this phenotype decreased with age). This made it difficult to determine whether a fly was on target and whether we could start the experiment by switching to the distractor. Memory tests were therefore only performed when the fly was on a straight, uninterrupted path to the landmark and crossing the midpoint of the arena with ±1 cm precision. Shapiro-Wilk tests were used to test for normality distribution and due to the data not being normally distributed, multiple comparison tests were then done using Kruskal-Wallis ANOVA (with in-built Bonferroni post-hoc). To compare the genotypes against the chance level, which is at 58% due to the flies showing a weak preference to turn to the opposite site after a previous turn (Neuser et al., 2008), the Sign test was used.

2.4. Tissue sections for electron microscopy and microtubules analysis

For electron microscopy, ultrathin Epon plastic sections were prepared as described in (Kretzschmar et al., 1997). TEMs were imaged with a FEI Morgagni 268 electron microscope. For double-blind analyses, images were taken and the pictures numbered. The measurements of microtubule area are described in (Bolkan and Kretzschmar, 2014), selecting neurites which contained microtubules that were cross-sectioned. No more than 5 neurites per image and at least 3 images per animal were analyzed. Three animals per genotype were used. The cross-sectional area of microtubules was measured with Photoshop before the genotype was revealed. The D'Agostino and Pearson omnibus test was used to test for normal distribution, followed by ANOVA one-way analysis of variance and Dunnett's post-tests.

2.5. Immunohistochemistry

For whole-mounts, brains were dissected in ice-cold PBS and transferred to 4% PFA in PBS. They were then fixed for 30 min to 1 h at room temperature (RT) and washed four times with PBS/0.5% Triton (PBS-T) for 10 min each before blocking with 5% normal goat serum in PBS overnight at 4 °C. To detect GFP, an antibody from Thermo Fisher Scientific (A-11122) was used at 1:250 overnight at 4 °C. Brains were then washed three times in PBS, 15 min each at RT and the secondary antibody applied (anti-rabbit-Cy2, Jackson ImmunoResearch) at 1:250 for 2 h at RT or overnight at 4 °C. Brains were washed three times for 10 min with PBS and mounted in Glycergel for confocal imaging using an Olympus FluoView 300 laser scanning confocal head mounted on an Olympus BX51 microscope.

2.6. Analysis of the axonal PDF phenotype

To quantify the axonal phenotype, we analyzed the two brain hemispheres separately. Confocal images were numbered and put into three categories by a person blinded to the genotype. In the case of homozygous flies, the categories were; normal, thinner axonal bundle or less branched target area, fragmentation in the axonal bundle or target area. For heterozygous flies, the categories were; normal, weakly disorganized target area (short axonal extensions or less branching in the target area), and disorganized target area (with long extensions beyond the target area).

2.7. Activity and sleep

Two independent experiments with at least 15 adult males from each genotype were performed for 5d and 14d old flies. For 35d old flies, three independent experiments were performed. Males were kept individually in glass tubes containing diet in one end with a piece of yarn plugging the other end. Tubes were placed in Drosophila Activity Monitors (DAM) models DAM2 or DAM5 (Trikinitecs, Waltham, MA, United States) to measure locomotor activity. Activity counts were taken once every minute for three days of light/dark (12 h:12 h LD), followed by at least seven days of constant darkness (DD). Activity experiments were performed at 25 ± 1 °C and ~1000–1200 lx of fluorescence light during light phase. Analysis of rhythmicity, as measured by fast Fourier transform (FFT), and sleep were performed with ClockLab 6 (Actimetrics, Wilmette, IL, United States). Sleep bouts

were defined as a consecutive 5-min interval or longer in which no activity was detected. Indicated ages were the age at the beginning of the activity recording. The D'Agostino and Pearson omnibus test was used to test for normal distribution, followed by ANOVA one-way analysis of variance and Dunnett's pos*t*-tests. Comparison between two values were done with Student's t-test.

2.8. Western blots

To detect hTau, we followed our previous protocol described in (Cassar et al., 2020). Fly heads were dissected, homogenized in lysis buffer [150 mM NaCl, 1% DOC, 1% SDS, 50 mM Tris, 5 mM EDTA, 5 mM EGTA, 1% triton X-100, and protease inhibitors (Cell Signaling Technology 5872S), and treated with 70% formic acid before gel electrophoreses under reducing conditions. Mouse anti-tau 5 (Invitrogen MA5–12808) was used at a dilution of 1:200, using 1× casein (Sigma C7594) as a blocking buffer. A biotinylated secondary antibody (Vector Labs BA-2000) and Streptavidin-conjugated alkaline phosphatase (Vector Labs AK-6000) was then used following the manufacturer's protocol.

To analyze CSP and Synapsin levels, heads were homogenized in the same lysis buffer and the lysate was centrifuged at 10,000 ×*g* for 10 min at 4 °C before being mixed with LDS sample buffer. Samples were denatured at 95 °C for 5 min prior to electrophoresis. Membranes were blocked with 5% non-fat dry milk dissolved in 1× TBST before primary antibody detection. ECL SuperSignal West Pico Plus (ThermoFischer Scientific) was used to visualize bands. Antibodies were used at the following dilution: mouse anti-CSP (1:200; DSHB), mouse anti-Synapsin (1:500; DSHB), and mouse anti-GAPDH G-9 (1:1000; Santa Cruz sc-365,062). For the quantification of protein levels, the intensity of the CSP and Synapsin bands were measured and normalized to GAPDH using Fiji. Statistical analyses were done using Kruskal-Wallis ANOVA to compare multiple groups or with a Mann-Whitney test when comparing two groups after D'Agostino and Pearson omnibus tests showed that the number of values is too low for normality.

3. Results

Using the CRISPR/Cas9 genomic editing system, we generated knock-in lines in which *Drosophila* Tau (dTau) was replaced by human Tau (hTau). In addition to wild-type hTau ($hTau^{WT}$), we selected two mutant forms of hTau (all in the 1N4R background) associated with Frontotemporal Dementia (FTD); $hTau^{V337M}$ and $hTau^{K369I}$. The correct insertion and expression of $hTau^{WT}$ and $hTau^{V337M}$ has been described previously (Cassar et al., 2020). We also confirmed the insertion of $hTau^{K369I}$ by PCR (not shown) and Western blots and as previously shown for $hTau^{V337M}$, this revealed increased levels of the mutant protein, possible due to increased accumulation (Supp. Fig. 1). We also confirmed a similar expression pattern of hTau and dTau by immunohistochemistry (Supp. Fig. 1). To detect recessive as well as dominant phenotypes caused by these mutations, we analyzed homozygous and heterozygous knock-in flies, whereby the latter were generated by crossing the knock-in lines to wildtype Canton S (CS).

3.1. Disease-associated Tau mutations cause progressive locomotion and memory deficits

As mentioned above, FTD patients can exhibit movement defects and we therefore tested our flies for impaired locomotion using the fast phototaxis assay, which assesses walking speed after a startle response. Homozygous $hTau^{V337M}$ flies showed a significant decrease in locomotion when only 3d old compared to CS (Fig. 1A, asterisks) or $hTau^{WT}$ (plus sign), whereas $hTau^{K369I}$ flies were not different from both controls at this age. When aged to 14d, $hTau^{WT}$ and $hTau^{K369I}$ also performed worse than CS control flies however, this did not reach significance (Fig. 1B). In contrast, $hTau^{V337M}$ flies were significantly impaired when compared to 14d old CS or $hTau^{WT}$. Aging to 28d further reduced the performance of $hTau^{V337M}$ and at this age $hTau^{WT}$, and $hTau^{K369I}$ also performed significantly worse than CS (Fig. 1C, asterisks). However, $hTau^{V337M}$ flies were still more impaired than $hTau^{WT}$ (plus signs) or $hTau^{K369I}$. Testing heterozygous flies, we did not detect changes in the fast phototaxis assay when 3d or 14d old (Fig. 1D, E) but $hTau^{V337M}/+$ performed worse when 28d old compared to age-matched CS or heterozygous $hTau^{WT}/+$ flies (Fig. 1F). $hTau^{K369I}/+$ flies were not different from the controls but significantly different from $hTau^{V337M}/+$ flies ($p < 0.001$). Together, this suggests that the V337M mutation, which is associated with FTDP-17, does cause locomotion deficits in agreement with the Parkinson's-like motor symptoms described in FTDP-17 patients, while the K369I mutation does not.

To determine whether our knock-in lines show memory defects, we used a short-term working memory assay. In this assay, walking flies must remember the position of a previously presented landmark and turn back towards it after a short distraction (Neuser et al., 2008). Using homozygous flies, we did not detect significant differences between the mutant hTau flies and control flies when 3d old and although the 14d-old mutant hTau lines performed worse than controls at 14d of age, this did not reach significance (Fig. 2A, B). All the lines were significantly different from chance level (indicated by the horizontal line and the plus signs below the boxes). When aged to 21d, both $hTau^{V337M}$ and $hTau^{K369I}$ flies performed significantly worse than controls and their mean was not significantly different from chance level (Fig. 2C). When heterozygous, 3d-old $hTau^{WT}/+$ and $hTau^{V337M}/+$ flies performed worse than CS or $hTau^{K369I}/+$ (possibly a developmental effect) but all lines were much better than chance level, revealing that they remembered the previous landmark (Fig. 2D). At 14d of age, both heterozygous mutant hTau flies showed memory deficits whereas $hTau^{WT}/+$ heterozygotes were not different from CS (Fig 2E). Determining the significance values to chance level revealed however that $hTau^{V337M}/+$ and $hTau^{K369I}/+$ flies still retained some memory. When aged for three weeks $hTau^{V337M}/+$ and $hTau^{K369I}/+$ had severe memory deficits and performed at random (Fig. 2F) whereas $hTau^{WT}/+$ flies were still not different from CS. Together, this shows that aged heterozygous mutant flies do show behavioral phenotypes in contrast to heterozygous $hTau^{WT}$, confirming that both mutations have dominant effects on memory. In contrast to the locomotion deficits (Fig. 1), homozygous mutant hTau flies did not exhibit the memory phenotype earlier, suggesting that heterozygosity for mutant hTau is as deleterious for cognition as having two copies of mutant hTau.

3.2. hTau causes disruptions in sleep patterns

We previously showed that $hTau^{V337M}$ flies have a disrupted sleep pattern as seen by an increase in the number of sleep bouts and a decrease in their length, a phenotype indicative of sleep fragmentation (Cassar et al., 2020). We therefore also analyzed the sleep pattern of $hTau^{K369I}$ flies. Counting the number of sleep bouts per day and their length, we did detect a significant increase in the sleep bout number while the length was decreased in 5d-old homozygous $hTau^{K369I}$ flies but this also occurred in $hTau^{WT}$ (Fig. 3A). When 14d old, similar changes were found in $hTau^{WT}$ but the $hTau^{K369I}$ flies more closely resembled CS (Fig. 3B). In contrast, at 35d of age both hTau lines were again indistinguishable from each other but showed a significantly fragmented sleep pattern compared to CS (Fig. 3C). We also tested whether the changes in sleep could be connected to an altered circadian activity pattern and found that while young flies showed no change, the circadian rhythm was less robust in the 35d-old $hTau^{K369I}$ flies but not in age-matched $hTau^{WT}$ flies (Supp. Fig. 2). Analyzing the sleep pattern of heterozygous flies, we did not detect significant changes in 5d and 14d-old flies with the exception of an increase in sleep bout length in 14d old $hTau^{K369I}/+$ compared to both CS and $hTau^{WT}/+$ flies (Fig. 3D, E). When 35d old, $hTau^{K369I}/+$ flies showed an increase in the number of sleep bouts compared to $hTau^{WT}/+$ while their length was decreased (Fig. 3F), therefore showing a fragmented sleep pattern. Analyzing rhythmicity in heterozygous flies did not reveal any changes (Supp. Fig. 2).

3.3. hTau mutations cause axonal defects

To determine what may cause the behavioral deficits, we expressed GFP in the PDF (Pigment dispersing factor) neurons. We focused on the PDF neurons for two reasons; they are involved in regulating sleep and changes in their morphology or connectivity could therefore be a cause for the sleep defects. Second, there are only eight PDF neurons in each hemisphere and their localization and axonal pattern is well described. The four large ventrolateral PDF neurons (called 1-LNv) in each brain hemisphere project to the contralateral side (arrowhead, Fig. 4A) whereas the four small ventrolateral neurons (s-LNv) send their axons to the dorsomedial protocerebrum where they synapse with their targets (arrows, Fig. 4A) (Helfrich-Forster et al., 2007; Tomioka and Matsumoto, 2009). Expressing mCD8-GFP (Lee and Luo, 1999) *via Pdf*-GAL4 revealed the normal pattern in CS and homozygous $hTau^{WT}$ when 5d old (Fig. 4A, B), including the projections of the s-LNvs and their termination in the dorsomedial protocerebrum (Fig. 4E, F). Accordingly, in both genotypes the majority of projections appeared normal (Fig. 4Q, light colors in the bars), although we judged some as showing a less complex target area (Fig. 4Q, medium colors). While most of the projections in 5d old homozygous $hTau^{V337M}$ and $hTau^{K369I}$ flies (Fig. 4C, D) also seemed normal, some appeared thinner and their terminations were less complex (arrows, Fig. 4G, H) and we also observed axonal fragmentation in a few flies (Fig. 4Q, dark color). When aged for 30d, the target area in both CS and homozygous $hTau^{WT}$ flies (Fig. 4J, N) appeared less organized than in younger flies but we did not observe any fragmentation (Fig. 4R). In contrast, in almost a quarter of the analyzed brain hemispheres of 30d-old homozygous $hTau^{V337M}$ we detected axonal fragmentation (arrowheads, Fig. 4K and Fig. 4Q). 30d old homozygous $hTau^{K369I}$ flies (Fig. 4L, P) revealed even more fragmented axons and none of the analyzed hemispheres showed a normal pattern (Fig. 4R). Together with the detectable axonal blebbing (arrowheads, Fig. 4O, P, magnification

in inset), which is a typical sign of axonal degeneration, this suggests that the mutant hTau proteins have lost the function of Tau in supporting axonal integrity.

Expressing GFP in PDF neurons in heterozygous flies, all genotypes had a similar percentage of normal and modestly affected axonal patterns of the s-LNvs when 5d old (Fig. 5A–D, Q). $hTau^{WT}$/+ flies also appeared very similar to CS when 30d old (Fig. 5E, F) with both genotypes showing a few elongated axons (Fig. 5R). In contrast, only a minority of the hemispheres in 30d-old $hTau^{V337M}$/+ and $hTau^{K369I}$/+ flies showed a normal axonal pattern (Fig. 5R) while the majority revealed a disrupted target area (arrow, Fig. 5G, H) and axons that extended far beyond the target field (arrowhead in H). To determine whether these axonal changes may be caused by effects of the mutations on the microtubule network, we expressed tubulin-GFP *via Pdf*-GAL4. As expected, 5d-old CS flies showed the normal projection pattern (Fig. 5I) and so did 5d-old $hTau^{WT}$/+ flies (Fig. 5J). In contrast, we now observed axons that extended beyond their target area already in 5d old flies heterozygous for $hTau^{V337M}$ or $hTau^{K369I}$ (arrowheads, Fig. 5K, L). In addition, we observed areas along the axons where the tubulin staining was weaker (arrows), suggesting a disrupted microtubules network. When aged to 30d, CS and $hTau^{WT}$/+ also showed somewhat extended projections compared to 5d-old flies (Fig. 5M, N) but this phenotype was less prominent than in 30d-old flies heterozygous for the mutant hTau genes (arrowheads, Fig. 5O, P). The disruptions in the projection pattern in the hTau mutant flies were more severe when expressing tubulin-GFP compared to mCD8-GFP which, together with the observed effects in the controls, suggests that the expression of the tagged tubulin promotes the axonal defects in $hTau^{V337M}$/+ and $hTau^{K369I}$/+.

3.4. Flies homozygous for the Tau mutations show an impaired microtubule structure

The experiments described above show that homozygosity for mutant hTau interferes with axonal integrity, possibly by having effects on microtubule stability. The mutated residue in $hTau^{V337M}$ is localized in exon 12, affecting the fourth microtubule-binding domain (R4) and *in vitro* experiments showed that the mutation reduces the ability of $hTau^{V337M}$ to bind and stabilize microtubules (Hasegawa et al., 1998; Hong et al., 1998). K369I is a point mutation at the border of the R4 domain and recombinant $hTau^{K369I}$ showed a reduced ability to promote microtubule assembly (Neumann et al., 2001). We therefore used electron microscopic preparations to detect effects of the mutations on microtubules *in vivo.* Measuring the cross-sectional area of microtubules in 30d old homozygous flies, we did not detect significant changes in any of the genotypes (data not shown). When aged to 60d, homozygous $hTau^{WT}$ also showed no difference compared to CS (Fig. 6A, B, E). However, both $hTau^{V337M}$ and $hTau^{K369I}$ flies showed an increase in the cross-sectional area of microtubules compared to both control lines (Fig. 6C–E). Stability of microtubules is not only regulated by microtubule-binding proteins but also by acetylation of tubulin (Saunders et al., 2022). We therefore tested whether the change in microtubules structure in the mutants could be due to effects on tubulin acetylation. As shown in supplementary fig. 3, using Western blots we did not detect significant changes in the levels of tubulin, acetylated tubulin, or the ratio of these two, further supporting that the structural changes in microtubules in the hTau mutant flies are due to a loss of the microtubules-associated function of Tau. Repeating the microtubules measurements with

30d- and 60d-old heterozygous flies, we did not detect significant changes (data not shown). The increase in microtubules suggest that the mutations impair the function of Tau in stabilizing microtubules, supporting the *in vitro* data. However, this phenotype only manifest when the flies are homozygous for hTau^{V337M} and hTau^{K369I}. It is likely that these changes in microtubules and the cell cytoskeleton are causing the observed axonal degeneration in the homozygous hTau mutants.

3.5. hTau^{K369I} shows reduced levels of the synaptic proteins Synapsin and CSP

We previously showed that hTau^{V337M}/+ flies show an increased number of synapses during the night by expressing a GFP-tagged end-binding protein 1 (EB1) construct in the PDF neurons (Cassar et al., 2020). EB1 is needed for microtubules to grow at their plus ends and it is also required for synapse formation (Akhmanova and Steinmetz, 2010; Conde and Cáceres, 2009; Stone et al., 2008). PDF neurons establish new synapses during the day and reduce them during the night, a process called synaptic homeostasis (Tononi and Cirelli, 2006; Tononi and Cirelli, 2014). However, this day/night adaptation does not occur in hTau^{V337M}/+ flies. Expressing EB1-GFP in the PDF neurons in hTau^{K369I}/+ flies did not reveal a difference in synapses (as measured by the number of EB1-GFP positive loops), neither during the day nor during the night (Supp. Fig. 4). We therefore addressed whether the hTau mutants may affect neuronal function by altering the levels of the synaptic proteins Synapsin and Cystein-String protein (CSP). Synapsin is a presynaptic protein required for maintaining synaptic transmission and its loss has been shown to cause learning and memory defects (Diegelmann et al., 2013; Klagges et al., 1996). CSP is attached to the membranes of synaptic vesicles, regulating vesicular exocytosis (Bronk et al., 2005; Gundersen et al., 2010). Measuring the levels of Synapsin in Western blots from head homogenates of homozygous flies, we did not detect significant differences, neither when 5d old nor when 30d old (Fig. 7A, B). Similarly, we did not find significant changes in the levels of CSP (Fig. 7C, D). Heterozygosity for the hTau mutations also had no effect on Synapsin levels in 5d old flies but at 30d we found a reduction in hTau^{K369I}/+ when compared to hTau^{WT}/+ flies by a pairwise comparison. In contrast, the Synapsin levels were not significantly different between in a pairwise comparison between hTau^{WT}/+ and hTau^{V337M}/+ (Fig. 7E, F, I). Measuring CSP levels, we again did not detect changes in 5d old flies but also found a decrease in its levels when comparing 30d-old hTau^{WT}/+ and hTau^{K369I}/+ flies whereas hTau^{V337M}/+ was not different from hTau^{WT}/+ (Fig. 7G, H, J). Together, this suggests that hTau^{K369I} interferes with synaptic transmission while hTau^{V337M} affects synaptic remodeling.

4. Discussion

Although several transgenic hTau *Drosophila* models were previously established using the GAL4/UAS system, our novel models are to our knowledge the first transgenic lines in which hTau was knocked into the endogenous dTau locus. This approach allows hTau to be expressed under the spatial and temporal regulation of the endogenous dTau promoter, therefore this more closely represents the normal conditions. The importance of a more physiological expression is underscored by findings from various groups that even the expression of wildtype hTau results in disease-associated phenotypes, although in general

they are weaker when compared to hTau mutations (Jackson et al., 2002; Wittmann et al., 2001). It has also been shown that hTau insertion lines with higher expression levels were more toxic than lines with lower expression levels (Jackson et al., 2002; Povellato et al., 2013), suggesting that increased levels of wildtype hTau are pathogenic. Consequently, it has been difficult to determine the role of disease-causing mutations in the development of specific phenotypes. Furthermore, these studies have been performed in the presence of endogenous dTau. which, besides raising the levels of total Tau, also prevents the development of recessive phenotypes that could contribute to the pathogenicity of disease-causing mutations. Although studies using these lines have provided many valuable results, our new models provide an additional approach to investigate the effects of disease-causing Tau mutations under more physiological conditions.

Analyzing our knock-in flies showed that the hTau mutations did have dominant effects but that some phenotypes could only be detected in homozygous flies. In general, we found that the phenotypes were stronger and occurred earlier in homozygous flies. In contrast, heterozygous flies had to be aged to reveal phenotypes. In the phototaxis tests and sleep studies with homozygous flies, we observed that even $hTau^{WT}$ performed worse than CS. This suggests that $hTau^{WT}$ may not be able to completely replace endogenous dTau and that these phenotypes are the consequence of a loss of dTau function. This may either be due to species-specific differences or because the human Tau constructs only encode one isoform that might not be sufficient to replace all dTau isoforms. Like in humans, the *Drosophila* gene encodes several dTau isoforms (14 protein isoforms are annotated in flybase; FlyBase Gene Report: Dmel\tau), including isoforms that vary in the number of microtubules binding sites. However, homozygous $hTau^{V337M}$ were still significantly different from homozygous $hTau^{WT}$ when aged. Together with the finding that heterozygous $hTau^{V337M}$ flies performed worse than heterozygous $hTau^{WT}$ or CS, whereas, $hTau^{WT}/+$ did not perform worse than CS, indicates that this mutation causes locomotion deficits in a dominant fashion. This is comparable to human patients, which are in general heterozygous and also develop symptoms with age. Interestingly, $hTau^{K369I}/+$ flies had no detectable effect in the phototaxis which is noteworthy because the $hTau^{V337M}$ variant is causing FTDP-17 which is characterized by motor defects similar to Parkinson's disease. In contrast, the $hTau^{K369I}$ mutation leads to Pick's disease, which does not cause Parkinsonism. In contrast, memory deficits are characteristic for both diseases and both mutations lead to short-term working memory defects in our models. Furthermore, $hTau^{WT}$ did not affect memory even when homozygous, indicating that this is a strictly dominant phenotype and not an effect due to the loss of dTau. It should also be mentioned that memory defects are already detectable in 14d-old flies whereas the other behavioral changes are only detectable when the flies are older (28-35d).

To identify possible mechanisms leading to the behavioral deficits in hTau mutant flies, we first addressed effects on Tau's best-described function; the interaction with and stabilization of microtubules. As mentioned above, both mutations reside in or close to the fourth microtubule-binding domain and *in vitro* both interfere with Tau's role in interacting with microtubules. Our analysis of microtubule size in homozygous mutant hTau flies confirmed effects of the mutations on microtubules *in vivo.* The increase in size in aged hTau mutants suggests that the mutant Tau interferes with the maintenance

of microtubule and not their composition (which is also supported by the normal tubulin/acetyl-tubulin levels). In agreement with a destabilization of the microtubules network, the homozygous mutant flies showed increased axonal blebbing and axonal fragmentation of PDF neurons that increased with aging. Although we did observe a few hTau mutant flies that showed axonal fragmentation already at 5d, the majority did not and even in the flies that did show fragmentation, the axons had initially grown into the target area. We therefore assume that the development and outgrowth of the PDF neurons is not affected. Although we did not detect changes in microtubule structure in 30d-old flies, more subtle changes in the microtubule network could lead to cytoskeletal changes that interfere with neuronal function, causing behavioral changes in younger flies (like the reduced locomotion in 3d- and 14d.old flies) and the axonal degeneration observed in 30d-old homozygous hTau mutant flies. Although we did not detect significant effects on microtubules size in heterozygous mutant hTau flies, we did detect disruptions in the tubulin staining pattern when expressing tubulin-GFP. It is therefore possible that more subtle effects on microtubules in heterozygotes may contribute to the phenotypes observed in heterozygous flies and possibly patients.

In contrast to the axonal degeneration, we observed in homozygous flies, heterozygous mutant hTau flies showed no phenotype when 5d old and aberrant axonal projections of PDF neurons and disruptions in their target area when aged. The PDF neurons play an important role in circadian rhythms and we previously showed that heterozygous $hTau^{V337M}$ flies do show significant changes in their sleep pattern (Cassar et al., 2020). We now show that the sleep pattern is also disrupted in aged $hTau^{K369I}/+$ flies, as seen by the increase in the number of sleep bouts and their decreased length compared to $hTau^{WT}/+$. Sleep fragmentation is also observed in patients with several neurodegenerative diseases including tauopathies (Borges et al., 2019; Holth et al., 2017; McCarter et al., 2016). In the case of $hTau^{V337M}/+$, the sleep disruptions seem to be caused by impaired synaptic homeostasis, because in contrast to CS and to $hTau^{WT}/+$, $hTau^{V337M}/+$ flies do not show a reduction of synaptic contacts during the night. When testing this in $hTau^{K369I}/+$ flies, we did not detect significant changes compared to CS or $hTau^{WT}/+$. We therefore tested whether we can detect other synaptic changes in $hTau^{K369I}/+$ flies. Measuring the levels of CSP and Synapsin, proteins which are both involved in synaptic transmission, we found no change in young flies but a decrease in 30d old $hTau^{K369I}/+$ flies, whereas $hTau^{V337M}/+$ was not different. This suggests that, although both hTau mutations affect synapses they do so by different mechanisms. While $hTau^{V337M}$ interferes with synaptic remodeling, $hTau^{K369I}$ impacts synaptic transmission. We did not detect changes in these synaptic proteins in homozygous flies, supporting our assumption that in these flies a loss of the microtubule-stabilizing function may be the major mechanism. We propose that in the case of the heterozygous mutant hTau flies, increasing defects in synaptic activity with age underlie the behavioral changes and may also lead to a remodeling of synaptic contacts as seen in the target area of the PDF neurons.

5. Conclusion

Using our novel hTau knock-in models, we showed that FTD-associated mutations interfere with microtubule morphology and with axonal integrity. While this confirms effects

of the mutation on the function of Tau in maintaining an intact microtubule network, effects on icrotubule stability were only significant in flies homozygous for the mutations. Nevertheless, heterozygous flies did show behavioral phenotypes, although they developed them only later in life. This is in agreement with dominant effects of these hTau mutations in patients because the vast majority of FTD patients are heterozygotes. In contrast to homozygous flies, heterozygous hTau mutant flies showed axonal targeting defects and synaptic changes. In summary, our results show that these FTD-associated Tau mutations cause a combination of dominant and recessive phenotypes. Due to the effects of both mutations on synapses when heterozygous, we propose that synaptic changes are a key factor in the pathogenicity in our models, suggesting this may also be the case in patients. However, it is possible that more subtle changes in microtubules contribute to the development of the disease. FTD develops over decades and even subtle effects on the microtubule network may eventually affect neuronal function and integrity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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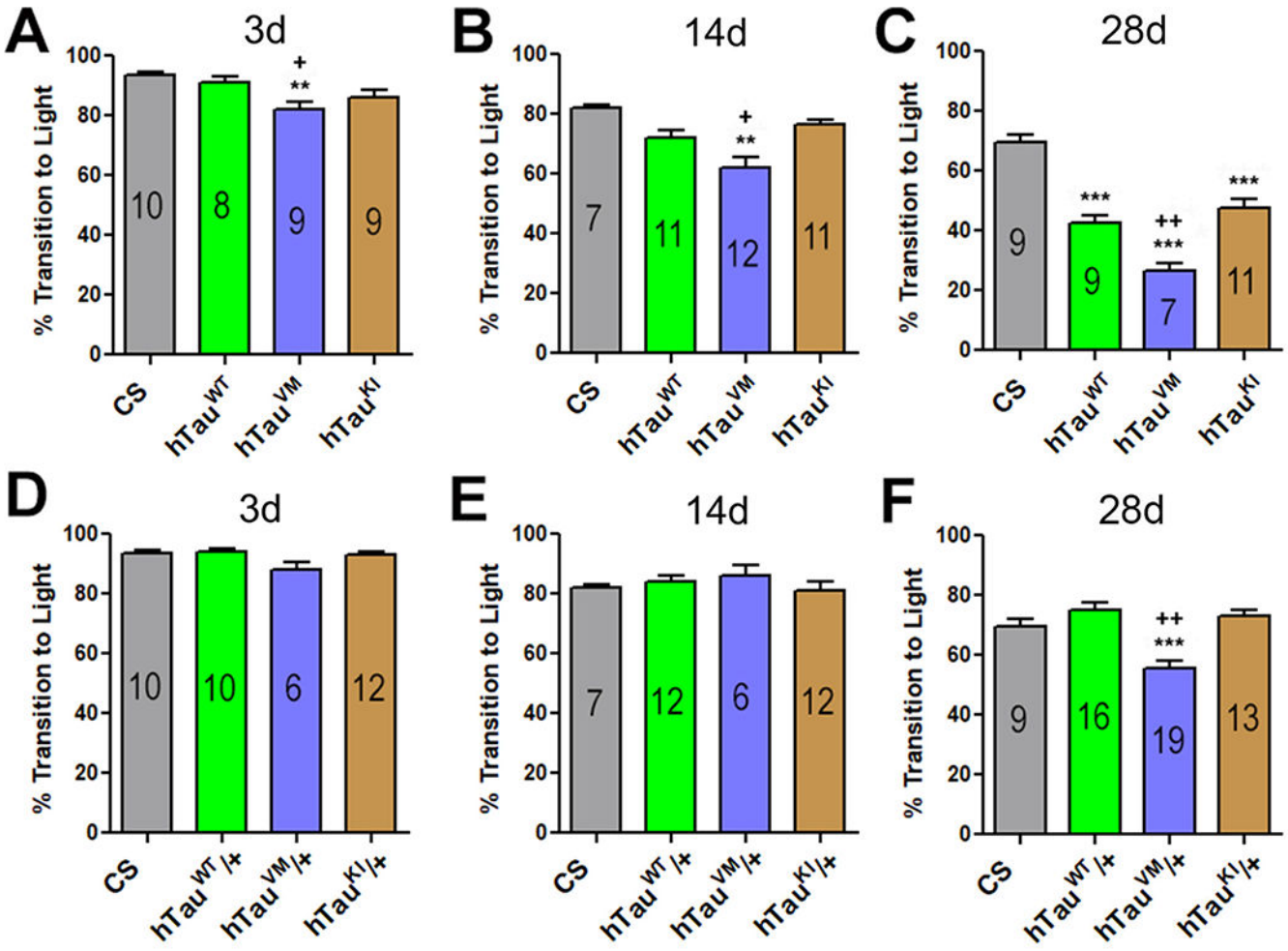

Fig. 1.

hTau flies show locomotion deficits. Homozygous $hTau^{V337M}$ flies perform worse in fast phototaxis assays when compared to CS (asterisks) or $hTau^{WT}$ (plus signs) when tested at 3d (A) or 14d (B) whereas homozygous $hTau^{K369I}$ flies are not significantly different from controls. When aged to 28d, all homozygous hTau flies perform worse compared to CS but $hTau^{V337M}$ is also significantly worse than $hTau^{WT}$ (C). When heterozygous, all hTau lines perform normal when 3d (D) or 14d old (E). When 28d old, $hTau^{V337M}$/+ flies perform significantly worse than the CS and $hTau^{WT}$/+ controls (asterisks) and $hTau^{K369I}$/+ (plus signs). Flies were tested in groups of 10–15 flies and the number of groups tested is indicated in the bars. Error bars indicate SEMs. Statistics was done with one way ANOVA and a Dunnett's post-test comparing to CS: $**p < 0.01$, *** < 0.001 or $hTau^{WT}$: $+p < 0.05$, $++p < 0.01$.

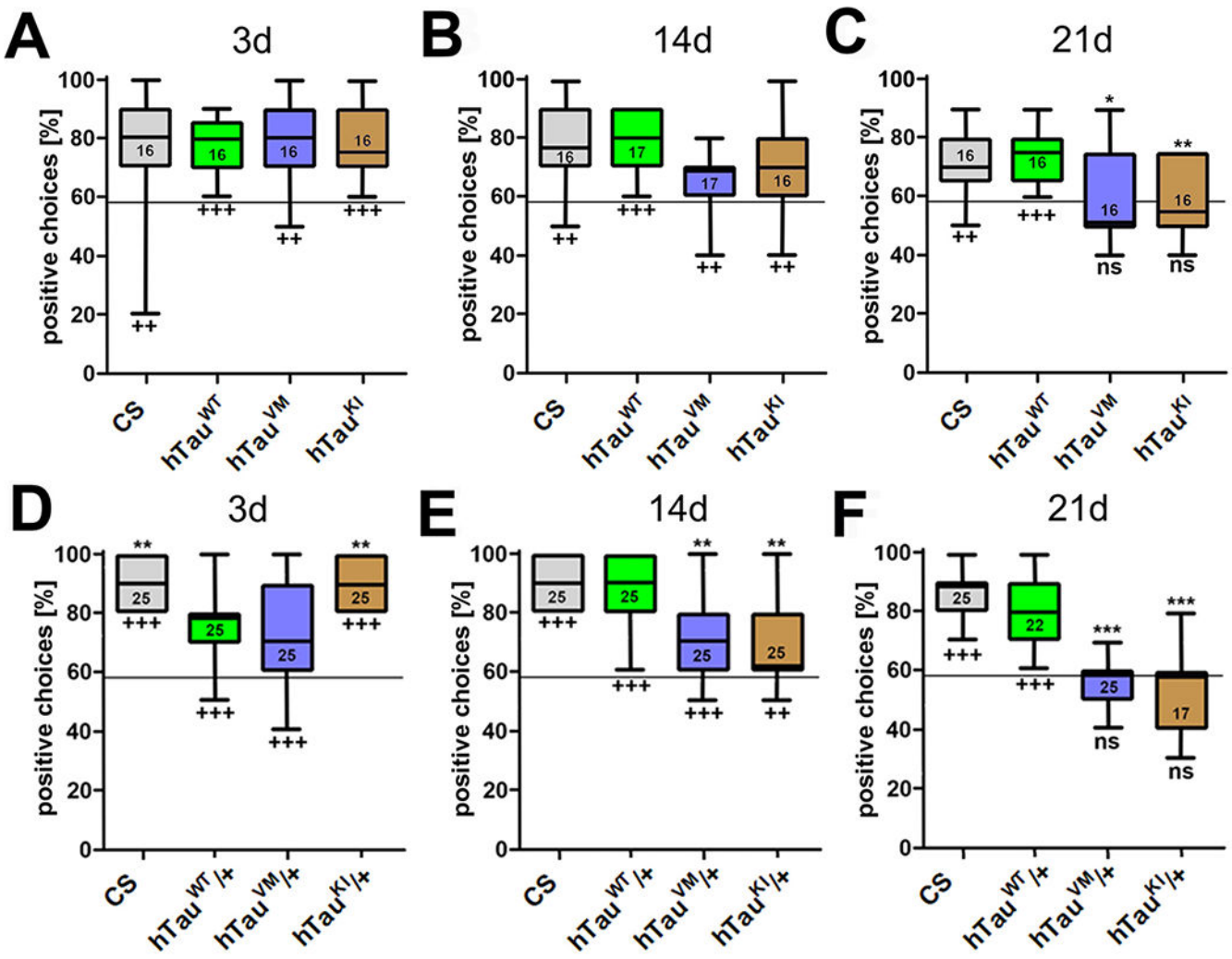

Fig. 2.

hTau flies show deficits in short-term working memory. A, B) Homozygous hTau^{V337M} and hTau^{K369I} flies do not show deficits in short-term working memory when 3d or 14d old but perform significantly worse when 21d old than hTau^{WT} (C, asterisks). Whereas the performance of the mutant hTau flies is still significantly different from chance level in the younger flies (plus signs), both hTau mutant lines perform randomly at 21d (ns). D) Heterozygous hTau^{WT}/T and hTau^{V337M}/+ perform worse than CS when 3d old but are much better than chance level. E) When 14d old, both hTau^{V337M}/T and hTau^{K369I}/T show a reduction in short-term memory compared to age-matched CS and hTau^{WT}/T. However, both still retain some memory when compared to chance level. F) At 21d of age, both mutant lines perform randomly whereas hTau^{WT}/+ still has an intact memory. Boxes denote the 25% and 75% quartiles, the horizontal lines the median, and the whiskers the 5% and 95% quantiles. Horizontal lines represent the chance level at 58% (due to the flies preferring the opposite direction from the last turn). Statistics was done using Kruskal-Wallis ANOVA. Sign tests were used to compare genotypes against the chance level. The number of tested

flies is indicated in the boxes. $p < 0.05$, *** < 0.001 compared to $hTau^{WT}$ or $hTau^{WT}/+$, respectively, ++$p < 0.01$, +++ < 0.001 compared to chance level, ns = not significant.

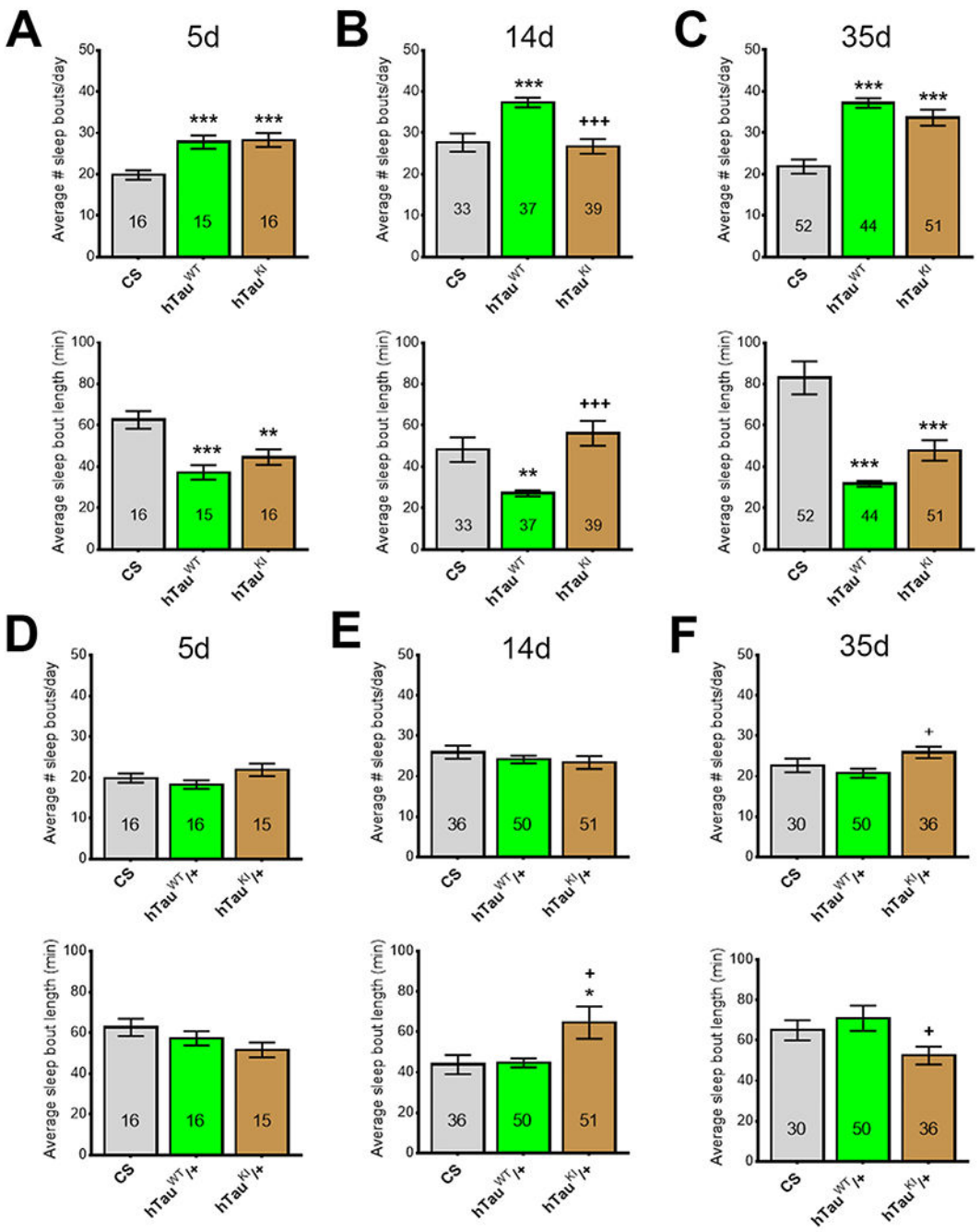

Fig. 3.

The sleep pattern is disrupted in hTau flies. A-C) Homozygous hTau flies have more sleep bouts per day but the length is reduced when compared to CS (with the exception of $hTau^{K369I}$ at 14d). No significant differences in sleep bout number and sleep bout length was detected in heterozygous hTau flies when 5d old but at 14d $hTau^{K369I}$ flies had longer sleep bouts compared to both CS and $hTau^{WT}/+$ (D, E). F) At 35d of age, sleep bout number is increased in heterozygous $hTau^{K369I}$ flies compared to $hTau^{WT}/+$ whereas sleep bout length is reduced. The number of flies used is indicated in the bars. Error bars indicate

SEMs. Statistics was done with one-way ANOVA and a Dunnett's post-test comparing to CS (asterisks) or $hTau^{WT}/+$ or $hTau^{WT}$ (plus signs). $^{**}p < 0.01$, *** < 0.001, $+p < 0.05$, $+++p < 0.001$.

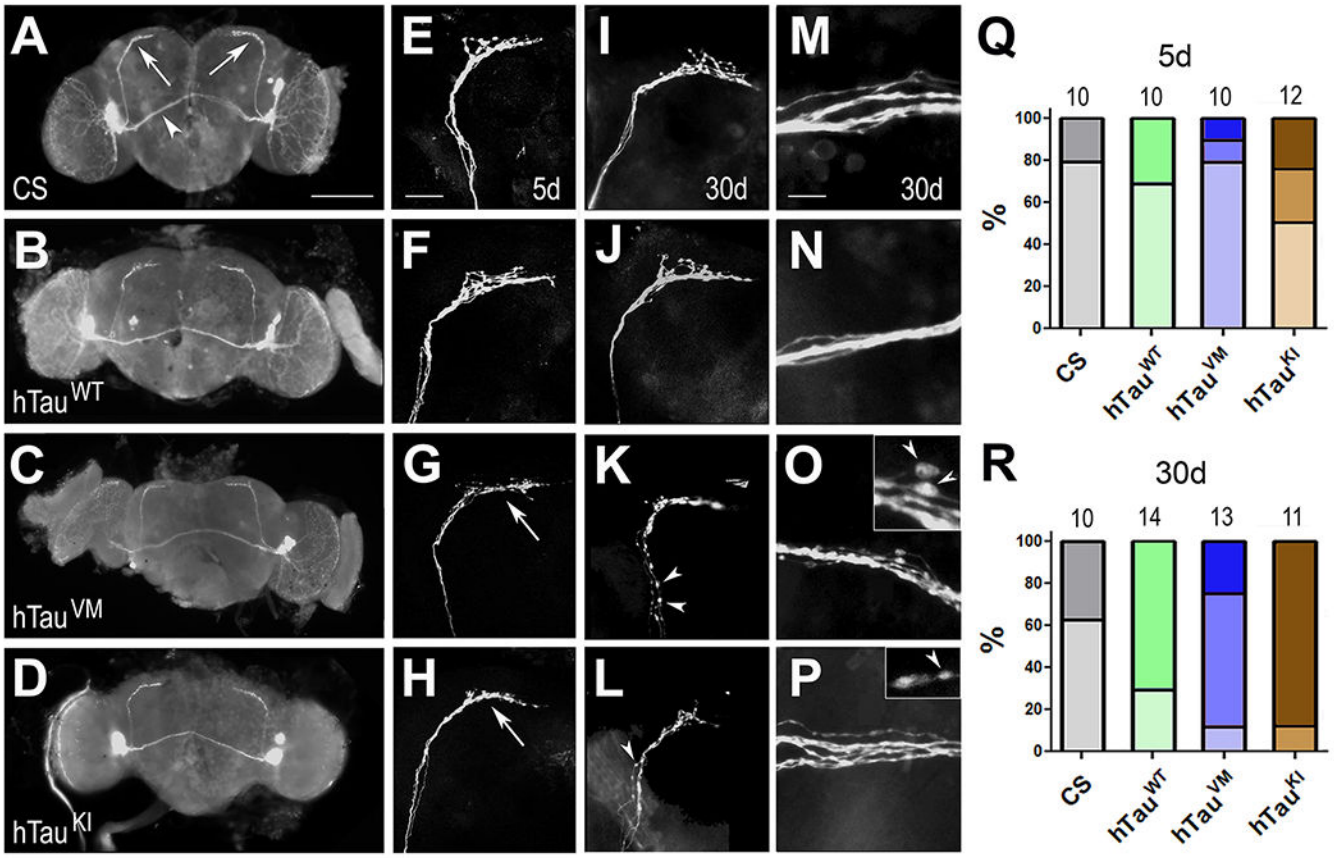

Fig. 4.

Homozygous mutant hTau flies show axonal degeneration when aged. A-D) Expression of mCD8-GFP with *Pdf*-GAL4 reveals the pattern of PDF neurons in 5d-old flies. Magnifications of the target area of the s-LNvs in the dorsomedial protocerebrum (arrowheads in A) shows the normal termination pattern in 5d-old CS and $hTau^{WT}$ (E, F). G, H) In 5d old $hTau^{V337M}$ and $hTau^{K369I}$ flies, the termination field appears less complex (arrows). In 30d-old flies, the pattern still appears normal in $hTau^{WT}$ when compared to CS (I, J) whereas the axons are fragmented (arrowheads) in $hTau^{V337M}$ and $hTau^{K369I}$ (*K,L*). M, N) Axons of the l-LNvs that project to the contralateral side in CS and $hTau^{WT}$. O, P) Fragmentation and axonal blebbing (arrowheads in the magnifications) is also seen in the axons of the l-LNvs in $hTau^{V337M}$ and $hTau^{K369I}$. Q, R) Percentage of brain hemispheres with a normal axonal pattern of the s-LNvs (light colors), with thinner or a less branched target area (medium colors), or fragmented axons (dark colors). The number of hemispheres analyzed is given above the bars. Scale bar in A = 100 μm, in E = 15 μm, in M = 5 μm.

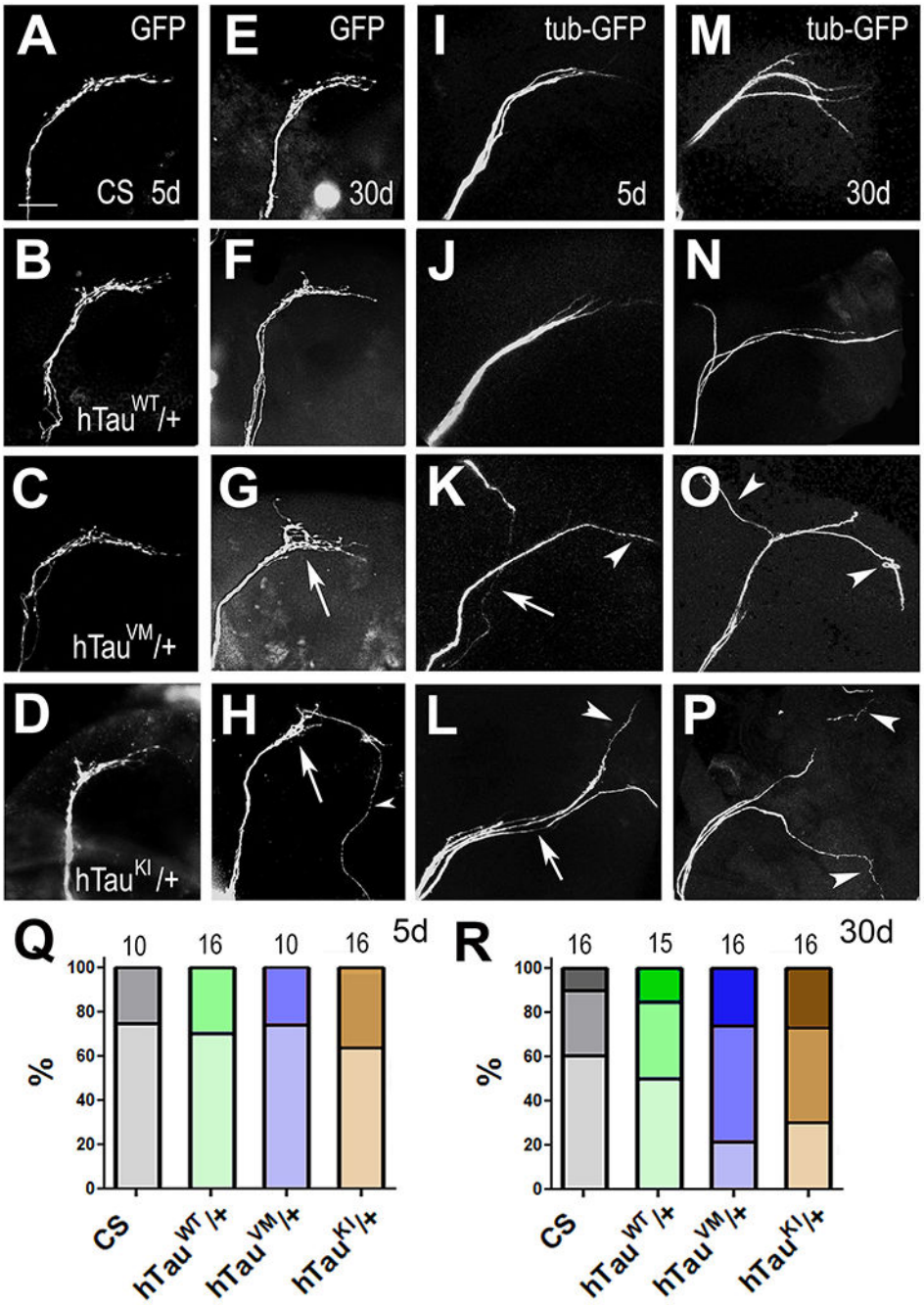

Fig. 5.

Disrupted axonal projections in heterozygous hTau flies. A-D) Expressing mCD8-GFP *with Pdf*-GAL4 did not reveal changes in the projection pattern in any of the 5d-old heterozygous flies. In contrast to the normal pattern in 30d-old CS and hTau^{WT}/+ (E, F), 30d old hTau^{V337M}/+ and hTau^{K369I}/+ flies show elongated and misrouted axons (arrowhead) and disrupted target fields (arrows, G, H). I, J) Expressing tubulin-GFP with *Pdf*-GAL4 reveals the microtubules network in 5d-old CS and hTau^{WT}/+ control flies. K, L). In 5d-old hTau^{V337M}/+ and hTau^{K369I}/+ flies, axons have extended beyond their target area

(arrowheads) and the tubulin staining appears weak and disrupted in some regions along the axons (arrows). In 30d-old CS and $hTau^{WT}/+$ flies expressing tubulin-GFP, the target area of the axons appears less constricted compared to 5d-old flies (N, M). This phenotype is much more prominent in 30d-old $hTau^{V337M}/+$ and $hTau^{K369I}/+$ with elongated and misrouted axons (O, P, arrowheads). Q, R) Percentage of brain hemispheres with a normal axonal pattern of the s-LNvs (light colors), with short axonal extensions or less branching in the target area (medium colors), and disorganized target area and/or long extensions beyond the target area (dark colors). The number of hemispheres analyzed is given above the bars. Scale bar in A = 20 μm.

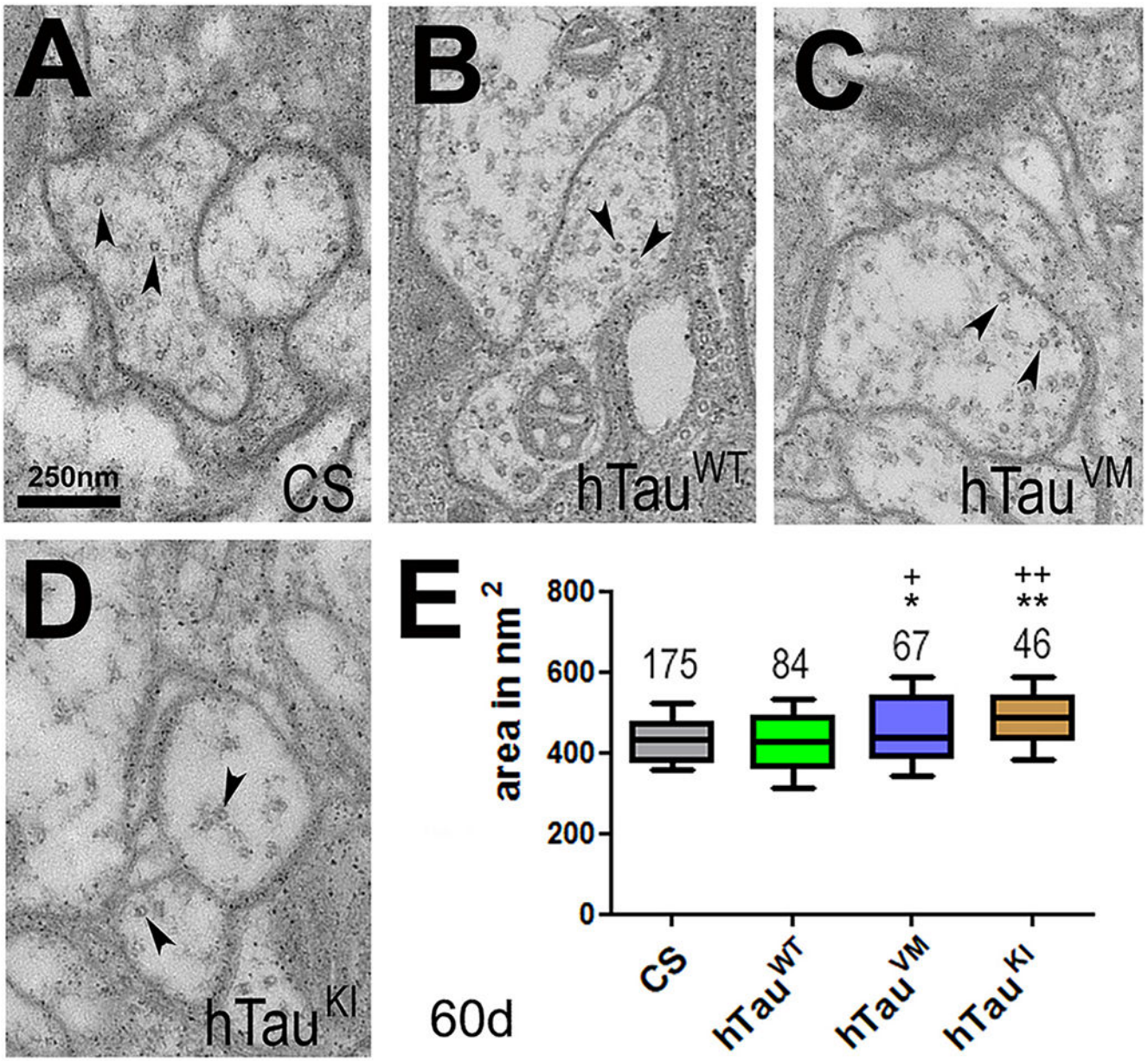

Fig. 6.

Mutant hTau affects microtubule morphology. A-D) Electron microscopic pictures from 60d-old flies (the arrowheads point to microtubules). E) Microtubule size, measured as cross-sectional area, is increased in both homozygous hTau mutants at 60d but not in $hTau^{WT}$. Microtubules were analyzed from 4 individuals for each genotype, with pictures taken in the medulla and deutocerebral neuropil. The number of microtubules measured is indicated above the boxes. Horizontal lines are medians; boxes are 25 and 75% quartiles; whiskers are 10 and 90% quantiles. Statistics was done with one-way ANOVA and a Dunnett's post-test to compare to CS $*p < 0.05$, $**p < 0.01$ or $hTau^{WT}$ + $p < 0.05$, $++p < 0.01$.

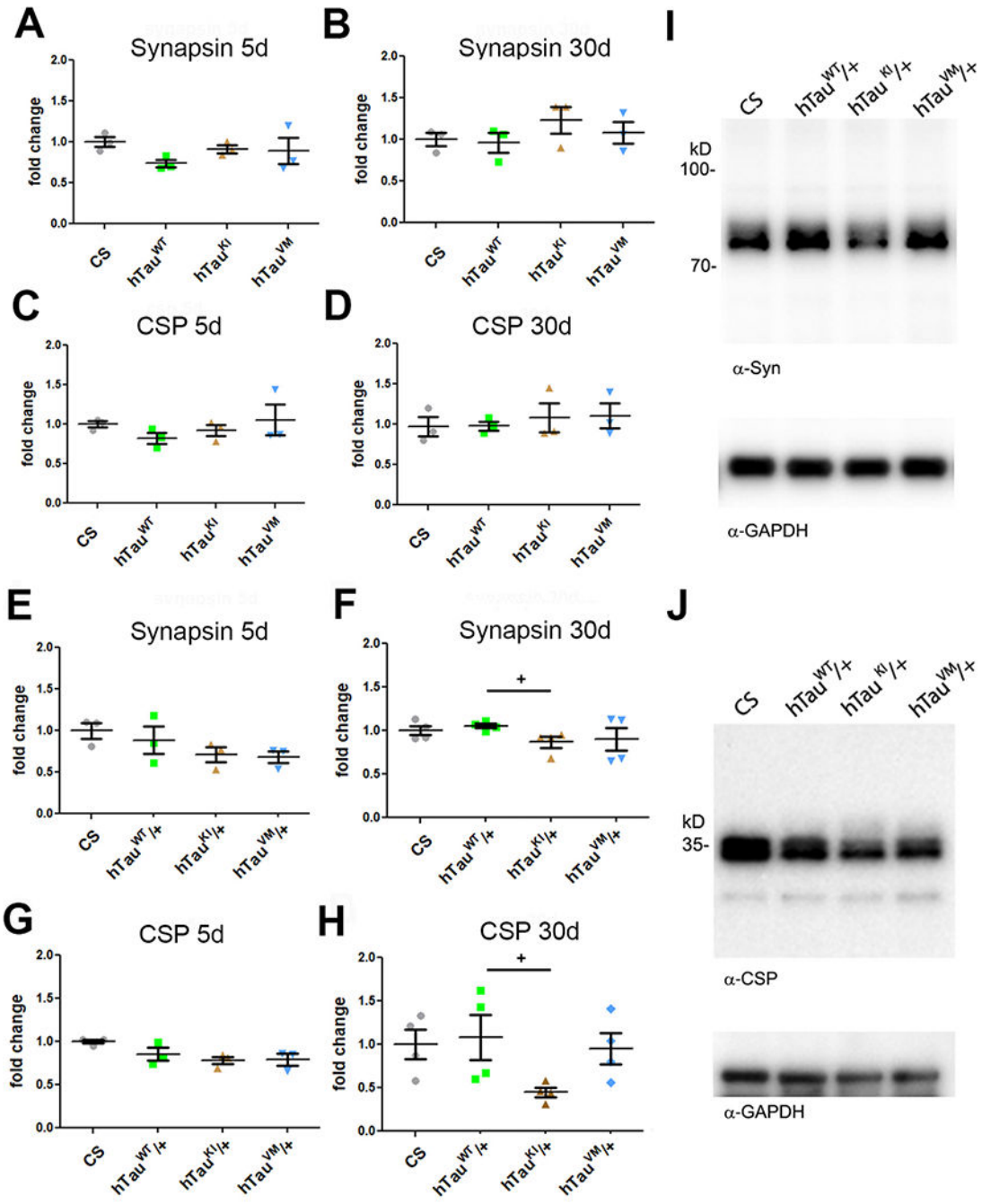

Fig. 7.

hTau^{K369I}/+ reduces the levels of synaptic proteins. A, B) Quantifying the levels of Synapsin, no significant change is seen in 5d- or 30d-old homozygous flies. C, D) CSP levels are also not significantly altered in 5d- and 30d-old flies. E, F) No effect on Synapsin levels is detectable in 5d old heterozygous flies but when 30d old, hTau^{K369I}/+ flies show a decrease in Synapsin. G, H) CSP levels are reduced in hTau^{K369I}/+ flies at 30d but not when 5d old. Representative Western blots showing Synapsin (I) or CSP (J) levels in 30d old flies. GAPDH was used as loading control, $n = 3$ with the exception of F, H were $n = 4$.

The horizontal line represents the mean and error bars indicate SEMs. Multiple comparisons were done using Kruskal-Wallis tests, pairwise comparisons between $hTau^{WT}$ and the two $hTau^{K369I}$ mutants were done with Mann Whitney tests ($+p < 0.05$).