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**TITLE:**

**Soluble hyper-phosphorylated tau causes microtubule breakdown and functionally compromises normal tau *in vivo***

**ABBREVIATED TITLE:** Soluble phospho-tau disrupts microtubules *in vivo*

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## ABSTRACT

It has been hypothesised that tau protein, when hyper-phosphorylated as in AD, does not bind effectively to microtubules and is no longer able to stabilize them, thus microtubules break down and axonal transport can no longer proceed efficiently in affected brain regions in AD and related tauopathies (tau-microtubule hypothesis). We have used *Drosophila* models of tauopathy to test all components of this hypothesis *in vivo*. We have previously shown that upon expression of human ON3R tau in *Drosophila* motor neurons it becomes highly phosphorylated, resulting in disruptions to both axonal transport and synaptic function which culminate in behavioural phenotypes. We now show that the mechanism by which the human tau mediates these effects is two-fold: firstly, as predicted by the tau-microtubule hypothesis, the highly phosphorylated tau exhibits significantly reduced binding to microtubules, and secondly, it participates in a pathogenic interaction with the endogenous normal *Drosophila* tau and sequesters it away from microtubules. This causes disruption of the microtubular cytoskeleton as evidenced by a reduction in the numbers of intact correctly aligned microtubules, and the appearance of microtubules that are not correctly oriented within the axon. These deleterious effects of human tau are phosphorylation dependent, because treatment with LiCl to suppress tau phosphorylation increases microtubule binding of both human and *Drosophila* tau and restores cytoskeletal integrity. Notably, all these phospho-tau mediated phenotypes occur in the absence of tau filament/ neurofibrillary tangle formation or neuronal death, and may thus constitute the mechanism by which hyper-phosphorylated tau disrupts neuronal function and contributes to cognitive impairment prior to neuronal death in the early stages of tauopathies.

**KEYWORDS:** Alzheimer's disease, tauopathy, axonal transport, lithium, neurofibrillary tangles.

## INTRODUCTION

It has been speculated that the axonal microtubular cytoskeleton may be compromised in Alzheimer's disease (AD) and other tauopathies because the microtubule associated protein tau, which usually stabilises this cytoskeleton, is abnormal in these conditions. In all tauopathies tau is atypically hyper-phosphorylated, misfolded and aggregated into filaments and tangles (reviewed in [20]). The idea that some of these aberrations of tau may result in defects in cytoskeletal integrity first came forth when it was demonstrated that microtubule assembly, in an *in vitro* assay, was defective in extracts from AD brains when compared to those obtained from age matched control brains [19]. It was shown that this was not due to dysfunctional tubulin proteins in the AD brains, but due to the hyper-phosphorylated state of the tau protein which, the authors speculated, affected its microtubule binding properties [14]. Subsequently, various studies analysed the effect of tau phosphorylation on its microtubule binding function, and showed that hyper-phosphorylation of tau reduces its binding to tubulin *in vitro* [4, 12, 27, 41] leading to a collapse of cytoskeletal integrity of cells in culture [10, 25, 26]. These studies have collectively led to the formulation of the "tau-microtubule" hypothesis which proposes that when tau is hyper-phosphorylated as it is in AD, it dissociates from microtubules which then collapse, and all neuronal functions dependent on the intact microtubular cytoskeleton, such as axonal transport and ultimately synaptic function, become disrupted [1, 13]. For the last few years this hypothesis has played an important role in putting forward a mechanism by which tau abnormalities, and in particular tau hyper-phosphorylation, may cause neuronal dysfunction and contribute to cognitive impairments prior to neuronal death in tauopathies. It is conceivable that it may depict the state of affairs within the AD brain because reduced microtubule numbers [5] and impairments in microtubule-based axonal transport have been reported in AD post mortem tissue [35, 39].

However, despite this body of circumstantial evidence that backs this hypothesis, it is hampered by the fact that it is almost exclusively based on *in vitro* findings with a dearth of *in vivo* supporting data. For instance, numerous studies have shown that expression of hyper-

phosphorylated tau in cells in culture disrupts their cytoskeleton and affects axonal transport [11, 28, 42], but it is not clear whether this holds true *in vivo*. The results from one rodent model of tauopathy that did attempt to study the effects of tau hyper-phosphorylation on cytoskeletal integrity and axonal transport *in vivo* was confounded by the fact these experiments were conducted on a background of neurodegeneration and neuronal death (which directly affects cytoskeletal integrity) making it difficult to delineate the causal role played by tau phosphorylation on these processes [21].

We have tested this hypothesis in a *Drosophila melanogaster* model of tauopathy in which we have expressed the highly phosphorylated shortest isoform (0N3R) of human tau (htau). This *Drosophila* model has enabled us, in this and previous studies, to test all the predictions made by the tau-microtubule hypothesis in one *in vivo* system, and to address the causative role played by tau hyper-phosphorylation on neuronal processes like cytoskeletal integrity and axonal/synaptic function in the absence of confounding neuronal degeneration. In our previous studies we have shown that, as proposed by the tau-microtubule hypothesis, expression of highly phosphorylated tau leads to profound impairments in axonal transport [30] and synaptic function [7]. The present study provides ultra-structural evidence showing, *in vivo*, that in the presence of highly phosphorylated human tau the integrity of the axonal microtubules breaks down. We further demonstrate that this effect is mediated by two pathogenic effects of the highly phosphorylated human tau: reduced ability to bind to microtubules itself, and physical interaction with and sequestration of the normal endogenous *Drosophila* tau thus compromising its microtubule-binding function.

## MATERIALS AND METHODS

*Flies*: Female *Drosophila melanogaster* expressing the GAL4 drivers GAL4-elav or GAL4-D42 were crossed with male flies transgenic for human 3-repeat tau under the UAS promoter (htau), or

with wild-type Oregon-R male flies (wt). Flies were raised on standard fly food with or without 20 mM LiCl.

*Electron Microscopy:* Filleted preparation of whole L3 larvae were fixed in primary fixative comprising 3% glutaraldehyde, 4% formaldehyde in 0.1 M PIPES buffer, pH 7.2 for a minimum of 1 hour. Specimens were rinsed in 0.1M PIPES buffer, post-fixed in 1% buffered osmium tetroxide (1 hour), rinsed in buffer, block stained in 2% aqueous uranyl acetate (20 min), dehydrated in an ethanol series and embedded in Spurr resin (Agar Scientific, Stansted, UK). The base of the ventral cord was marked on the block using a dissecting microscope and gold/silver sections were cut through the nerves at this level on a Leica OMU 3 ultramicrotome. The sections were stained with Reynolds lead stain and viewed on a Hitachi H7000 transmission electron microscope equipped with a SIS Megaview III digital camera. For quantification of microtubule numbers, the number of microtubule profiles per axon was counted in cross sections of peripheral nerves. Comparable regions (spanning the same segmental areas) of peripheral nerves emerging from the same proximal points of exit from the ventral cord in all animals were studied. Within this, all visible axons of the peripheral nerves were analysed in 5 animals per condition (approximately 240 axons per animal) with the experimenter blinded to condition. Different genotypes/treatment groups were processed in parallel to avoid experimental artefacts resulting from differential processing.

*Microtubule-binding assay:* For each condition, 6 heads of 1 day-old flies or 10 brains of L3 larvae were pooled and homogenized in 40  $\mu$ l microtubule-binding assay buffer containing protease, kinase and phosphatase inhibitors (100 mM MES pH 6.8, 500  $\mu$ M Mg SO<sub>4</sub>, 1 mM EGTA, 4 mM DTT, 2 mM dGTP, 20  $\mu$ M taxol, 0.1% triton-X 100, 30 mM NaF, 20 mM sodium pyrophosphate, 40mM 2-glycerophosphate, 3.5 mM sodium orthovanadate, 10  $\mu$ M staurosporine, and protease inhibitor cocktail). Homogenates were centrifuged at 12,000 g for 1 hour at 4°C, the pellet representing the fraction enriched for microtubule-bound proteins. Assays were repeated in 5 independent experiments.

*Immunoprecipitation:* For each condition, 16 heads of 1 day-old flies were pooled and homogenized in 500  $\mu$ l of microtubule-binding assay buffer (as above), and the microtubule-bound fraction was pelleted as described above. This was done to pre-clear microtubules from the homogenates so that the immunoprecipitation would be carried on the non-microtubule-bound supernatant, in order to eliminate any co-precipitation between tau molecules which arose simply from their mutual binding to microtubules. *Drosophila* tau (dtau) was then immunoprecipitated from the supernatant as follows: pre-clear with Protein A/G plus-agarose beads (Santa Cruz) for 1 hour at 4 °C; incubation with 4  $\mu$ l anti-dtau (St. Johnston) for 1 hour at 4 °C; and incubation with protein A/G beads for 1 hour at 4 °C. The supernatant from this incubation was kept, as well as the beads which were washed 3 times with buffer lacking dGTP and taxol.

*Western blotting:* Samples were heated for 5 minutes at 95 °C in Laemmli buffer, separated by PAGE according to standard methods, and transferred to PVDF membrane by semi-dry transfer. Blots were probed with the following primary antibodies: anti-human tau (Dako, 1:15,000), anti-dtau (1:500, St. Johnston), anti-phospho-tau (PHF-1, 1:2000), and anti-tubulin (Developmental Hybridoma Bank, 1:200). Signal was detected using fluorescently conjugated secondary antibodies and a LiCor scanner with Odyssey software. Resulting band densities were measured using Image J software.

*Larval Dissections for immunohistochemistry* Late 3<sup>rd</sup> instar larvae were dissected in ice-cold hemolymph-like 6 (HL6) saline. Each larva was cut along the length of the dorsal midline, the digestive tract and trachea were removed and the CNS exposed.

*Immunocytochemistry* Dissected larvae were fixed in 4 % paraformaldehyde buffered in HL6 for 90 minutes at room temperature. Tissues were then washed in phosphate buffered saline (PBS) plus 0.1% Triton X-100 (PBSTX) for 10 minutes and blocked in a solution containing 5% normal rabbit serum, 3% horse serum, 2% bovine serum albumin and 0.2% PBSTX for 120 minutes, before co-incubation with rabbit anti-dtau (St Johnston, 1:1000) and mouse anti-htau (Abcam UK 1 in 1000) overnight at 4 °C, on a rotator. Skins were then washed with PBSTX for 15 minutes and then co-

incubated in FITC-conjugated anti-rabbit and Texas Red-conjugated anti-mouse secondary antibodies. Skins were washed with PBSTX for 15 minutes, cleared using glycerol and mounted in Vectashield (Vector Laboratories).

*Imaging* All specimens were visualized on a Zeiss 510 meta confocal microscope using an oil immersion lens plan-apochromat (63X 1,4 oil DIC) to collect a Z-stack series image through the tissue.

## RESULTS

### **Axon microtubule cytoskeleton breaks down following expression of highly phosphorylated human tau**

We have previously shown that the human tau (htau) we express in *Drosophila* is highly phosphorylated at a number of sites including AT8, PHF-1, 12E8 [30], and AT100 (Sealey, Cowan and Mudher *unpublished observations*). The tau-microtubule hypothesis predicts that hyper-phosphorylated tau has reduced ability to bind to microtubules and as a result the microtubule cytoskeleton breaks down. To test whether this mechanism is responsible for the axonal transport disruption we reported in htau-expressing *Drosophila* [30], we used transmission electron microscopy (EM) to examine transverse sections of peripheral nerves of fly larvae to analyse the organisation of the microtubules in axons. We found that expression of highly phosphorylated htau was associated with compromised axonal microtubule integrity. This was evident in two ways: first there was a significant reduction in the number of microtubule profiles oriented correctly with the main axonal axis in htau-expressing axons. Typically in transverse section the microtubules appear as characteristic circular structures of 25nm in diameter (Fig. 1). Second, in htau-expressing axons there was considerable evidence of microtubules as if cut obliquely and often in random orientations, rather than transversely. These oblique microtubule profiles were often in the same axons that also contain apparently clean transverse section of microtubules. In sections of peripheral nerve axons of control larvae expressing GAL4-elav driver alone (wt), transverse profiles of



regularly spaced microtubules were observed (black arrows in Fig. 1a and supplementary Fig. 1a-c). In contrast, in larvae expressing htau which is highly phosphorylated, there were markedly fewer such transverse microtubule profiles, with some axons exhibiting a handful of microtubule profiles per section, whilst others lacked any such profiles (black arrows Fig. 1b and supplementary Fig. 1d-f). Quantification of these profiles in over one hundred sections confirms that there were significantly fewer transversely oriented microtubule profiles in htau-expressing axons compared to controls (7.8  $\pm$  0.2 microtubule profiles were visible per axon cross-section in control larvae, 4.5  $\pm$  0.2 per axon in tau larvae - Fig. 1d,  $p=0.0001$ ) and that the majority of axons in the htau-expressing larvae contained less than 5 transverse microtubule profiles whilst the majority of axons in control larvae typically contained more than 6 intact microtubule profiles (supplementary Fig. 2). Instead, in the htau-expressing axons, there was the appearance of incorrectly aligned microtubules (white arrows in Fig. 1b and supplementary Fig. 1d-f). These structures are unlikely to represent microtubules cut longitudinally, because extreme care was taken to ensure that all sections were cut in the transverse plane and most htau-expressing axons contained such oblique profiles alongside transverse profiles of microtubules. This would suggest that the microtubule appearance in these axons cannot be simply because they were not cut in the transverse plane. To confirm that the compromised cytoskeletal integrity in htau-expressing larvae was mediated by the highly phosphorylated htau, we treated larvae expressing htau with lithium chloride (LiCl). LiCl is widely used to inhibit the GSK-3 $\beta$ -mediated phosphorylation of tau [24, 38], and we have previously shown that rearing htau-expressing flies on food containing 20 mM LiCl results in larvae in which tau phosphorylation is significantly reduced [30]. Indeed, LiCl intake by larvae resulted in a restoration of microtubule integrity as was evident by the appearance of greater numbers of transverse microtubule profiles in Li treated htau-expressing larvae (htau/Li) compared to those in untreated htau larvae (black arrows in Fig. 1c and supplementary Fig. 1 g-h). Quantitative analysis confirms that these differences were statistically significant: 4.5  $\pm$  0.2 microtubule profiles were visible per axon cross-section in h-tau larvae, and 6.5  $\pm$  0.4 in the htau/Li larvae ( $p<0.001$ ) (Fig.

1d). These results are unlikely to be attributable to poor fixation/processing procedures in the htau-expressing tissue because animals from each genotype/treatment group were processed in parallel in independent experiments and both the execution of this process as well as the quantification of EM sections was performed with the experimenter blind to the experimental condition. Furthermore in the htau-expressing larvae, the axons that displayed a reduced number of microtubule profiles still contained normal looking mitochondria and intact membranous structures opposing the likelihood that the collapsed microtubular integrity arose because of an experimental artefact. Moreover a minimum of five animals was analysed in each genotype/treatment group and all were seen to be representative of the phenotypes described above (supplementary Fig. 1 and supplementary Fig. 2).

Overall, these data indicate that expression of highly phosphorylated htau causes a significant disruption of the axonal microtubular cytoskeleton, and that the microtubules are restored to normal when phosphorylation of tau is prevented.

In addition to displaying compromised cytoskeletal integrity, we noticed that some of the htau-expressing axons had a larger diameter than control axons (12% of control axons had a diameter of more than 900 nm compared to 31% of htau-expressing axons). This enlarged diameter of axons in htau-expressing animals may reflect axonal swellings arising as a result of htau-mediated disruption to axonal transport [30].

Notably, filamentous structures were never detected in these axon cross sections (nor in biochemical assays – data not shown) indicating that these effects were mediated by soluble and not aggregated human tau.

### **Highly phosphorylated human tau and endogenous *Drosophila* tau exhibit impaired microtubule-binding ability**

An explanation for the loss of microtubule integrity in the presence of highly phosphorylated tau is, as predicted by the tau-microtubule hypothesis, a reduced binding of the phospho-tau to the microtubules. We explored the microtubule-binding properties of the htau

expressed in our model using a standard microtubule-binding assay. Assays were performed using extracts from both L3 larvae (as used for the EM studies described above), and 1d old adult flies. We found that in both adults (data not shown) and larvae, when highly phosphorylated htau was expressed, there was an absence of significant polymerized tubulin coming down in the microtubule-enriched pellet (compare the htau lane with wt in the MT pellet section of Fig. 2a). Quantification showed that approximately 10% of htau was bound to microtubules with the remainder appearing in the supernatant fraction (middle lane of SN blot in Fig. 2a and Fig. 2b). In animals treated with LiCl the binding of human tau to microtubules increased significantly to approximately 25% with a consequent reduction of htau immunoreactivity in the supernatant fraction (SN in Fig. 2a and Fig. 2b). These data shows that there is a inverse relationship between the phosphorylation state of the human tau, and its binding to microtubules and the integrity of the axonal microtubular cytoskeleton.

A feature of our results is that expression of highly phosphorylated htau was sufficient to cause microtubule collapse despite the presence of endogenous *Drosophila* tau (dtau). This implies that highly phosphorylated htau somehow interacts with and affects the microtubule-binding function of the endogenous dtau. To test this, we examined the microtubule-binding properties of the endogenous dtau in our model. Using antibodies that specifically react with dtau, we found that a significant amount of dtau was bound to microtubules in control animals that were not expressing htau. In larvae expressing highly phosphorylated htau, the microtubule-binding ability of dtau was reduced (Fig. 2a) by as much as 60% (i.e. only 20% of dtau was bound to microtubules in htau-expressing larvae compared to the 50% that is usually bound to microtubules in control larvae – Fig. 2c). This indicates that expression of highly phosphorylated human tau functionally compromised the microtubule-binding ability of the endogenous *Drosophila* tau.

The effects of htau we have demonstrated have been dependent upon its highly phosphorylated state and are rescued by LiCl treatment. We next asked if the effect of htau on the microtubule binding abilities of dtau were similarly phosphorylation dependent. Hence we

examined the microtubule-binding properties of dtau in larvae treated with LiCl. The results show that LiCl treatment significantly reduced tau phosphorylation (Fig. 2e) and significantly increased the percentage of dtau bound to microtubules from 20% to 40% in htau-expressing larvae (Fig. 2a, c). These findings indicate that human tau is able to dissociate *Drosophila* tau from microtubules *in vivo* in a phosphorylation-dependent manner.

### **Mechanism by which human tau affects *Drosophila* tau**

The finding that highly phosphorylated soluble htau functionally compromises normal endogenous tau is novel. One explanation for this is that by inducing a breakdown of cytoskeletal integrity and disruption of axonal transport, the highly phosphorylated htau prevents the delivery of the endogenous dtau to the axonal compartment where it can bind to the microtubules. We explored this possibility by using confocal microscopy to examine the distribution of the endogenous dtau in the axons of larvae that were expressing the highly phosphorylated htau. We found that both the highly phosphorylated htau and the endogenous dtau were both homogeneously distributed along the length of the axons, with no evidence of any retention of either protein within any neuronal compartment (supplementary Fig. 3). Furthermore the distribution of dtau in the htau-expressing animals was no different to that usually seen in wt controls (data not shown). Treatment of the htau-expressing animals with LiCl similarly did not disturb the cellular localisation of the endogenous dtau (supplementary Fig. 3) It is unlikely that the highly phosphorylated htau disrupts the normal distribution of the endogenous *Drosophila* tau.

It is conceivable that the htau expression leads to a down-regulation of the endogenous dtau expression and this is why there appears to be reduced microtubule binding by the endogenous tau in the presence of the htau. This is unlikely since there was no difference in the expression levels of dtau in htau-expressing animals compared to wild-type control animals (Fig. 3a, b).

Another possibility is that the highly phosphorylated htau may affect the phosphorylation status of the dtau. We have demonstrated above that the microtubule-binding ability of the htau is

dependent upon its phosphorylation state and that decreasing phosphorylation (by treating with LiCl) leads to an increase in microtubule binding. We therefore asked whether highly phosphorylated htau causes a reduction in the microtubule binding ability of the dtau by mediating an increase in its phosphorylation state. Antibodies specific for phosphorylated dtau are not currently available. We therefore indirectly examined the phosphorylation state of dtau in wt control flies, in htau flies and in htau/Li flies by incubating brain extracts with alkaline phosphatase (AP) and analysing gel motility using anti-tau antibodies. This makes the assumption that highly phosphorylated proteins would exhibit a retarded gel motility and undergo a significant shift and become faster after incubation with AP. In line with its highly phosphorylated nature, htau exhibited a retarded gel motility which became faster following treatment with AP (Fig. 3c). Dtau also exhibited a degree of phosphorylation in all animals because it too underwent a motility shift upon treatment with AP (Fig. 3c). However, the extent of the band shift that dtau underwent after treatment with AP did not differ between wt flies and those expressing htau (Fig. 4c upper panel), indicating that htau expression had no effect on the phosphorylation status of dtau. Furthermore, the motility of dtau was the same in brain extracts from wt animals compared to those from htau-expressing animals (Fig. 3c upper panel) confirming that htau did not induce hyper-phosphorylation of dtau. These findings collectively imply that dtau did not become more phosphorylated in the presence of highly phosphorylated htau.

One other possible mechanism by which htau could interact with dtau is that htau may form filamentous structures which could trap and sequester the dtau away from microtubules. Such an effect of human tau on endogenous rodent tau has been described in a rodent model of tauopathy [29]. However, it is unlikely that this is the mechanism by which the highly phosphorylated htau impacts the endogenous normal dtau in our model since we saw no evidence of filament formation either by EM or by biochemical analysis (data not shown).

In the absence of filament formation, it is still conceivable that the soluble/non-aggregated highly phosphorylated htau could physically interact with the endogenous dtau and siphon it away

from microtubules. We used immunoprecipitation assays to explore this possibility. Brain extracts from 1d old flies were first centrifuged to pellet out microtubules (Fig. 4a – third panel) (to avoid any interaction between htau and dtau arising because of mutual binding of the two to tubulin) and the supernatants were then incubated with an anti-dtau antibody. In all conditions, the dtau antibody pulled down dtau as one would expect, but in htau-expressing larvae, the dtau antibody also pulled down htau (Fig. 4a first and second panels). This suggests that dtau and htau physically interact with each other. To verify that this is the mechanism by which the htau interferes with the microtubule binding function of dtau, we compared the htau-dtau interaction in htau-expressing animals reared on control food and LiCl food. Treatment with LiCl reduced the htau-dtau interaction by nearly 50% (Fig. 4b) strongly suggesting that the restorative effect of LiCl may have resulted from reduced sequestration of dtau by the highly phosphorylated htau. This data demonstrates a novel pathogenic effect of soluble, non-aggregated highly phosphorylated human tau *in vivo*: it can physically interact with endogenous normal *Drosophila* tau and dissociate it from microtubules, thus functionally compromising it.

## DISCUSSION

### **Summary**

We describe here two pathogenic effects of highly phosphorylated human tau in a *Drosophila* model of tauopathy, which may reflect the role played by such abnormal tau proteins in AD and other tauopathies. Firstly, we show that, as predicted by the tau-microtubule hypothesis, highly phosphorylated tau exhibits reduced microtubule binding which leads to a loss of microtubule structural integrity *in vivo*. The second pathogenic effect of highly phosphorylated human tau is that it physically interacts with the normal endogenous *Drosophila* tau and “poisons” it - dissociating it from microtubules and functionally compromising it. All these phenomena are dependent upon the phosphorylation state of the human tau and not on tau filaments. Furthermore these findings provide a molecular mechanism for the human tau-mediated effects on neuronal

function that we have previously described: defects in axonal transport, synaptic transmission, and behaviour in this *Drosophila* model of tauopathy [7, 30].

This study underscores the validity of using *Drosophila* models such as this to dissect the cellular and molecular mechanisms that underlie early pathological changes in tauopathies. It provides the first *in vivo* demonstration of the entire sequence of events predicted by the tau microtubule hypothesis and in so doing sheds light on the mechanism by which hyper-phosphorylated tau plays a pathogenic role in tauopathies.

### **Expression of highly phosphorylated human tau results in breakdown of microtubules**

Tau protein has long been known to function as a microtubule-associated protein (MAP), and as such is able to bind directly to microtubules and stabilize them *in vitro* [8, 15, 37]. The tau protein in AD brains is hyper-phosphorylated at a number of sites [14, 48], and it is well documented that this phospho-tau has reduced ability to bind to and stabilise microtubules *in vitro* [2, 4, 12, 17, 27, 31, 32, 36, 41, 44, 45] and in cells [10, 23]. It has also been shown that as well as exhibiting impaired microtubule binding ability, soluble hyper-phosphorylated tau can interact with and compromise other normal microtubule associated proteins (including normal tau) in cell-free and cell culture based *in vitro* systems [1, 3, 23]. That this should lead to defects in the integrity of the microtubular cytoskeleton is conceivable since impairments in fast axonal transport (which relies on an intact microtubular cytoskeleton) have been shown *in vitro* [28] and *in vivo* [30] following tau over-expression. In another *in vivo* study in which human tau was expressed, several components of the tau-microtubule hypothesis have been described, namely: tau hyper-phosphorylation, reduced microtubule numbers, impaired axonal transport, and behavioural phenotype [21]. Although this study provides compelling evidence for some components of the tau-microtubule hypothesis, it is confounded by the fact that these observations were made at a time when significant axonal and neuronal degeneration was also occurring, making it difficult to identify the primary cause of the microtubule depolymerisation. Furthermore, while these studies demonstrate a correlation between

tau hyper-phosphorylation and reduced microtubule numbers and impaired axonal transport, a causative role of tau phosphorylation in mediating these effects has not been fully explored. The ultra-structural evidence presented in the present study is the first demonstration, to our knowledge, of the microtubule-disrupting effect of hyper-phosphorylated tau in an intact animal.

Our results highlight the causal role played by the phosphorylation state of tau in mediating this pathogenic effect on microtubule assembly *in vivo*. Further support for this concept comes from a rodent model in which it was shown that anaesthesia leads to acute phosphorylation of three, but not four, repeat tau, and that it is the former that becomes compromised in its ability to bind to microtubules whilst the latter remains bound [34]. Interestingly, FTDP-17 mutant tau has similarly been shown to be unable to promote microtubule assembly due to a heightened phosphorylation state [16], implying that the hyper-phosphorylated state of tau may play a pivotal role in the pathogenic mechanisms that underlie all tauopathies, genetic or sporadic. However, it is conceivable that phosphorylation at different sites of tau may have different pathogenic consequences, with GSK-3 $\beta$  sites regulating microtubule binding and thus phospho-tau-mediated neuronal dysfunction, whilst PAR-1 sites play a role in phospho-tau-mediated neuronal death. Support for this idea comes from studies in *Drosophila*, showing that human tau resistant to phosphorylation at GSK-3 $\beta$  sites exhibits increased microtubule binding but retains its toxicity, whilst human tau resistant to phosphorylation at the PAR-1 sites is no longer toxic even though it exhibits reduced microtubule binding [6].

### **Highly phosphorylated tau functionally compromises normal *Drosophila* tau.**

Our findings that highly phosphorylated tau compromises the biological function of normal tau is in line with the predictions made by the tau-microtubule hypothesis that both hyper-phosphorylated soluble and aggregated tau can sequester further normal tau away from the microtubules and thus exacerbate the ongoing abnormal tau-mediated disruption of microtubule integrity [18]. The sequestering ability of aggregated tau has recently been demonstrated in



transgenic mice in which the expression of an aggregate-prone fragment of mutant tau led to the formation of tangles that contained both the exogenous human tau and the endogenous rodent tau [29]. Soluble hyper-phosphorylated tau has also been shown to interact with recombinant normal tau and disrupt microtubule assembly in an *in vitro* cell-free experimental paradigm [3]. However, it has not yet been shown that abnormally phosphorylated non-aggregated tau can functionally compromise normal tau *in vivo*. We demonstrate this here by showing, for the first time, that the expression of phosphorylated soluble human tau alone can cause the dissociation of endogenous *Drosophila* tau from microtubules without forming paired helical filaments, and that normal binding can be restored by inhibiting phosphorylation. It is unlikely that this interaction of human tau with *Drosophila* tau is due to an unnatural interaction between two foreign tau proteins, because we have previously shown that over-expression of *Drosophila* tau (or indeed bovine tau) results in the same phenotypes in our model as those seen following over-expression of human tau [43, 46]. This suggests that over-expression of tau proteins from any species may mediate neuronal dysfunction by the same mechanism.

Our results imply that the ability of abnormal tau to functionally compromise normal tau is dependent upon its phosphorylation state and *not* its aggregation state. In trying to unravel the mechanism by which the human tau was functionally compromising the *Drosophila* tau, we demonstrated that the two proteins physically interact with each other *in vivo*. Interestingly, reducing the phosphorylation state of human tau, a treatment that increases microtubule binding of both human and *Drosophila* tau and restores microtubular integrity, results in reduced interaction between the two tau proteins. These results suggest that a significant pathogenic effect of soluble highly phosphorylated human tau is that it can interact with normal tau, sequester it away from microtubules, and thus compromise its ability to bind to and stabilise microtubules.

**What is the significance of tangle formation in tau mediated dysfunction?**

There is divided opinion in this field as to the role played by tangles in the pathogenesis of tauopathies. The tau-microtubule hypothesis predicts that following hyper-phosphorylation, the tau which has been displaced from the microtubules goes on to form filaments which ultimately coalesce into the neurofibrillary tangles which are observed in AD and other tauopathies. In various versions of the model, these filaments may themselves be cytotoxic; they may sequester away further tau from the microtubules, exacerbating the loss-of-function (as discussed above); or they may be an incidental downstream consequence. It is important, however, to distinguish between these possibilities, as much work has gone into elucidating the mechanisms of filament formation, and therapeutic strategies to prevent it. In human studies, loss of synapses and associated cognitive dysfunction precedes filament and neurofibrillary tangle formation in AD brains [9, 40]. In various rodent tauopathy models animals exhibit cognitive impairments and other disease phenotypes long before they develop neurofibrillary tangle pathology [21, 22, 33]. Similarly in the original *Drosophila* tauopathy models, flies never develop filaments or tangles and yet they undergo neurodegeneration [46, 47]. In this and a previous study we show that tau-mediated neuronal dysfunction is attributable to the phosphorylation state of tau prior to filament and tangle formation. We therefore conclude that therapeutic strategies might be better aimed at stabilization of microtubules and/or targeted modulation of tau phosphorylation, than at prevention or dispersal of filaments. This work strongly supports the growing opinion that significant neuronal dysfunction precedes overt neuro-pathological hallmarks.

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The authors declare that they have no conflict of interests.

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**Fig. 1****Over-expression of phosphorylated human tau is associated with disruption of the microtubule cytoskeleton**

Electron micrographs of transverse sections of peripheral nerves in L3 *Drosophila*. a) In WT larvae the axon profiles show numerous regularly spaced microtubule profiles (black arrowheads). b) In human tau (htau)-expressing axons the microtubules are dramatically disrupted: there are many fewer microtubule profiles evident (black arrowheads) and there is evidence of disorganised microtubules in the same axon profiles (white arrowheads). These observations are unlikely to be artefacts of the plane of section because the axons include microtubule profiles that are clearly transverse and microtubules profiles that are not transverse (see materials and methods for care taken to ensure correct orientation of sections). c) In htau-expressing larvae fed with 20 mM LiCl (htau/Li) there is a partial rescue with many more microtubule profiles evident (black arrowheads) and less evidence of misaligned microtubules. Scale bar = 200 nm. d) Control axons contained on average  $7.8 \pm 0.2$  visible microtubule profiles per cross-section, larvae expressing htau contained only  $4.5 \pm 0.2$ , and those expressing htau but reared on LiCl contained  $6.5 \pm 0.4$  profiles. Average numbers of visible profiles in control and htau/Li axons were each highly significantly different from those visible in htau axons ( $p < 0.01$ , unpaired students t-test). Scale bar = 500nm.

**Fig. 2****Human tau expression dissociates endogenous *Drosophila* tau from microtubules, and inhibition of tau phosphorylation by LiCl increases microtubule binding of both human tau and *Drosophila* tau**

Microtubule-binding assays were performed *ex vivo* using extracts from lines expressing driver alone (wt) or human tau (htau) or human tau and reared on media containing 20mM LiCl (htau/Li). Third instar larval brain extracts were fractionated to yield a microtubule pellet (MT) and a soluble supernatant (SN). Both fractions were probed with anti-human tau, anti- *Drosophila* tau (dtau) and anti-tubulin antibodies (a). Quantification of 5 such independent microtubule binding assays confirms that in the htau expressing lines, only a small percentage of the htau is bound to microtubules (a, b) and that the microtubule binding of endogenous dtau in these flies is also reduced when compared to controls (a, c). Treatment with LiCl significantly increases binding of both htau and dtau to microtubules (b, c). These results are not the product of unequal expression of tubulin because the total levels of tubulin are not significantly different between the three lines (d). Quantification of five independent western blots in which lysates from larval brains were probed with a phospho-tau antibody PHF-1 confirms that expression of htau in this model increases tau phosphorylation and treatment with LiCl decreases it (e). (\* indicates  $p < 0.05$  and \*\*  $p < 0.01$  student's paired t-test;  $n=5$ ).

**Fig. 3****Expression of highly phosphorylated human tau does not affect the expression levels or phosphorylation status of endogenous *Drosophila* tau**

Lysates from 1d old adult fly heads were probed with anti-*Drosophila* tau antibodies and showed that neither expression of human tau (htau) nor treatment with LiCl (htau/Li) alter the expression of *Drosophila* tau (dtau) when compared to controls (wt) (a). Quantification of three such independent experiments confirms this (b). Lysates were prepared from heads of 1d old flies expressing driver alone (wt) or htau. These were incubated with (+) or without (-) alkaline phosphatase (AP) for 1h at 37°C and then separated by SDS-PAGE and probed with anti-htau and anti-dtau antibodies. Treatment with AP caused a downward mobility shift of both the dtau (c, upper panel) and the htau (c, middle panel) indicating that both species of tau were constitutively phosphorylated though the greater downward shift of the htau compared to the dtau suggests that htau was more phosphorylated than dtau. The downward shift of dtau from lysates of wt flies was not different to that seen from lysates of flies expressing htau indicating that expression of htau does not significantly increase the phosphorylation status of the endogenous dtau.

**Fig. 4****Soluble human tau binds to *Drosophila* tau in a phosphorylation dependent manner.**

An Anti-*Drosophila* tau antibody was used to precipitate out *Drosophila* tau (dtau) from 1d old adult fly heads expressing either driver alone (wt) or human tau (htau) or human tau and reared on media containing 20mM LiCl (htau/Li). This lysate was probed with antibodies to dtau, htau and tubulin. Dtau co-immunoprecipitates with htau but treatment of the htau expressing flies with LiCl abolishes this interaction (upper panel in a). This interaction between the two species of tau is unlikely to be an artefact of mutual binding of the two proteins to tubulin because prior to the immunoprecipitation reaction, tubulin was pre-cleared from the lysates by pelleting it out and there was thus no tubulin present in these lysates (lowest panel in a). This result was confirmed in five independent experiments the averages of which are graphically presented in b.

**Supplementary Fig. 1**

**Over-expression of phosphorylated human tau disrupts cytoskeletal integrity.** Electron micrographs of transverse sections of peripheral nerves in L3 *Drosophila*. In WT larvae the axon profiles typically show 8 or more regularly spaced microtubule profiles (black arrowheads a-c). In human tau (htau) -expressing axons the microtubules are dramatically disrupted: the numbers of intact microtubule profiles (black arrowheads in d-f) are significantly fewer than those seen in wt axons and there is instead the appearance of disorganised microtubules in the same axon profiles (white arrowheads in d-f). Treatment of the htau-expressing larvae with LiCl (htau/LiCl) reverts the microtubule cytoskeletal integrity back to that seen in wt axons with more regularly spaced intact microtubule profiles evident (black arrowheads in g-i). Scale bar = 500nm.

**Supplementary Fig. 2****Percentage of axons exhibiting intact transverse microtubule profiles**

The numbers of axons displaying intact transverse microtubule profiles as assessed by EM were counted in larvae expressing elav driver alone (wt), or human tau (htau) or human tau and reared on 20mM LiCl (htau/Li). The majority of wt axons contain large numbers (over 5) of transverse microtubule profiles and very few axons contain no such profiles. In contrast the majority of htau expressing axons contain either none or very few (less than 5) such profiles. Treatment of the htau larvae with Li increases the numbers of axons containing large numbers of intact transverse microtubule profiles and reduces the numbers of htau axons with no such profiles.

**Supplementary Fig. 3****Human tau does not alter the cellular distribution of *Drosophila* tau**

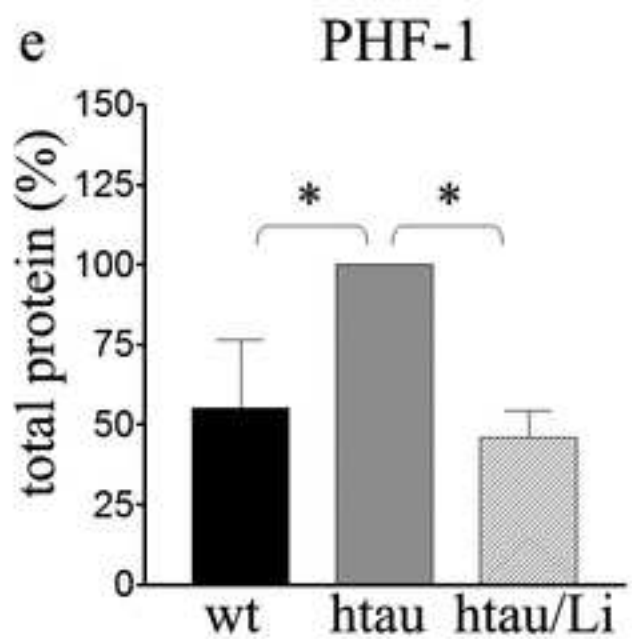
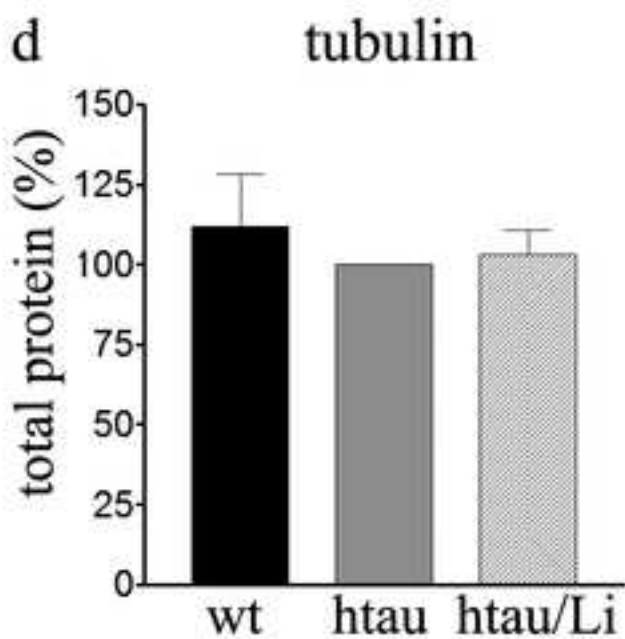
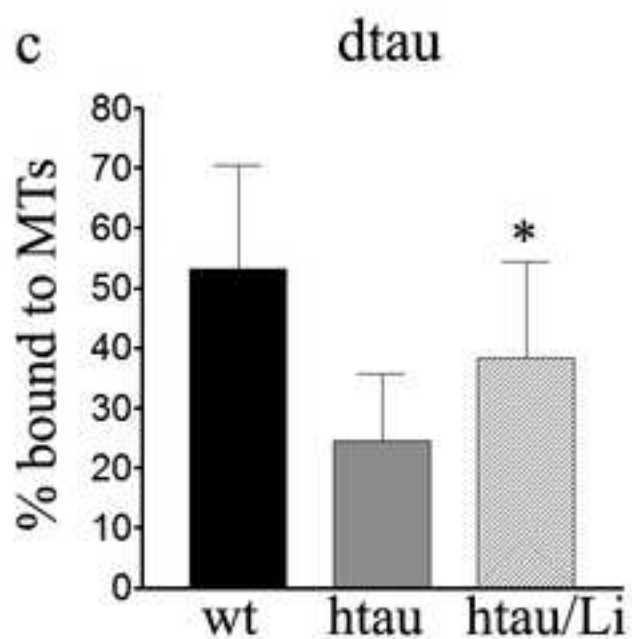
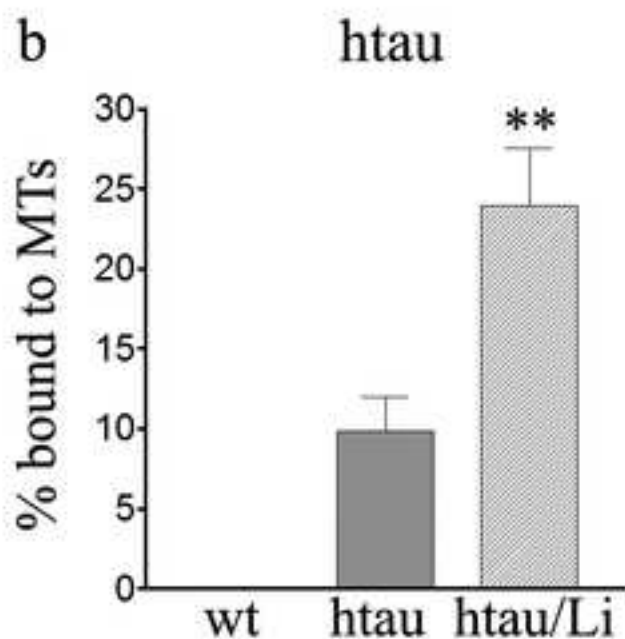
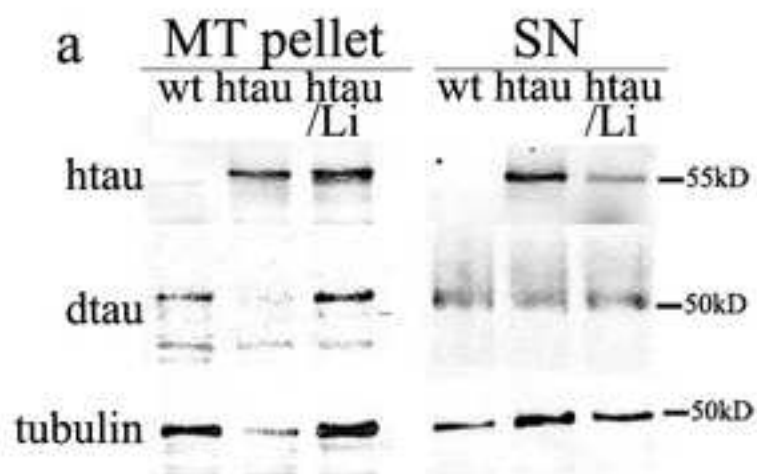
Confocal image analysis was used to study the neuronal distribution of human tau (htau) and *Drosophila* (dtau) in htau expressing animals that had been reared on normal food and food containing 20mM LiCl (htau/Li). Dtau was homogeneously distributed along the axons in wt (data not shown). Neither htau expression (a, b) nor treatment with LiCl (d, e) altered the distribution of dtau. There was significant co-localisation between htau and dtau (c, f). Scale bar = 15µm.

**Fig #1**

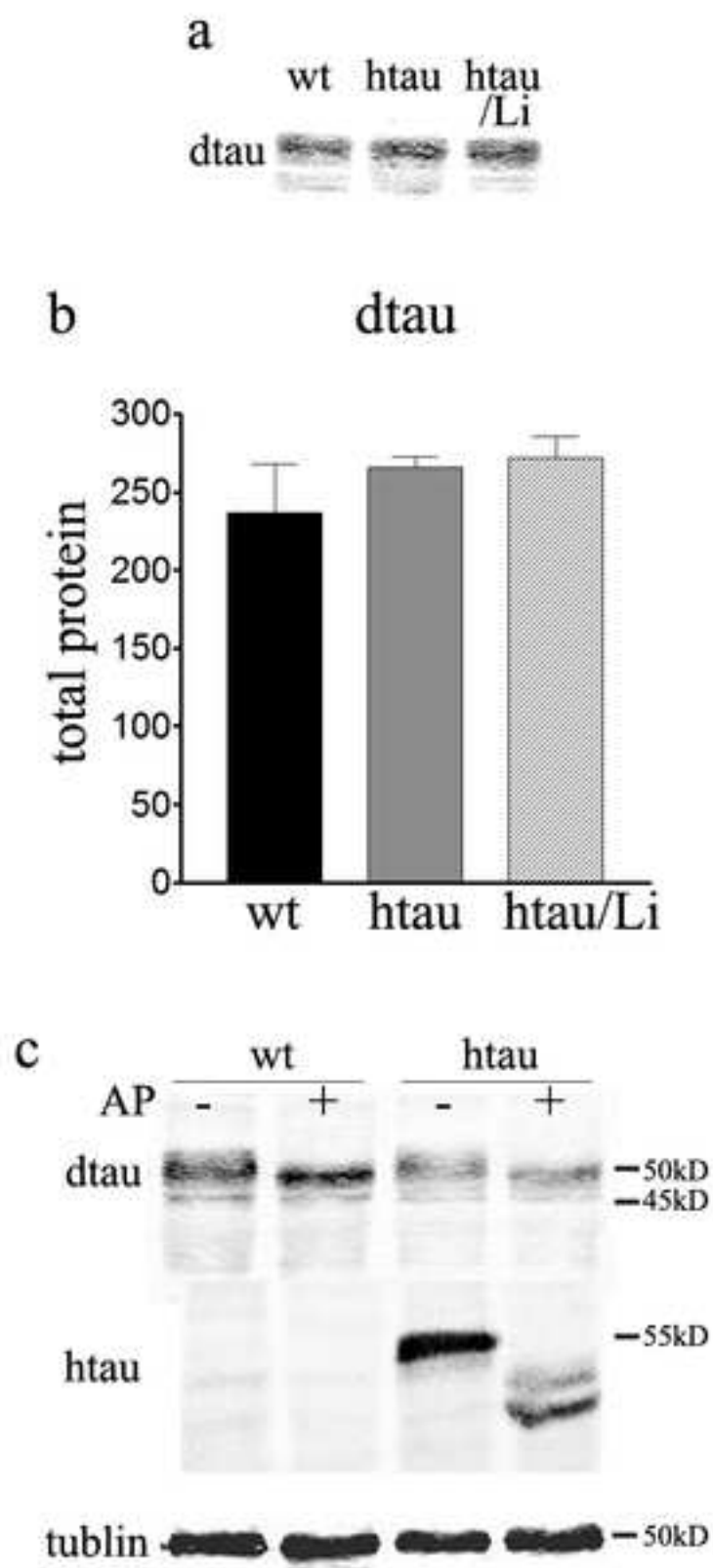
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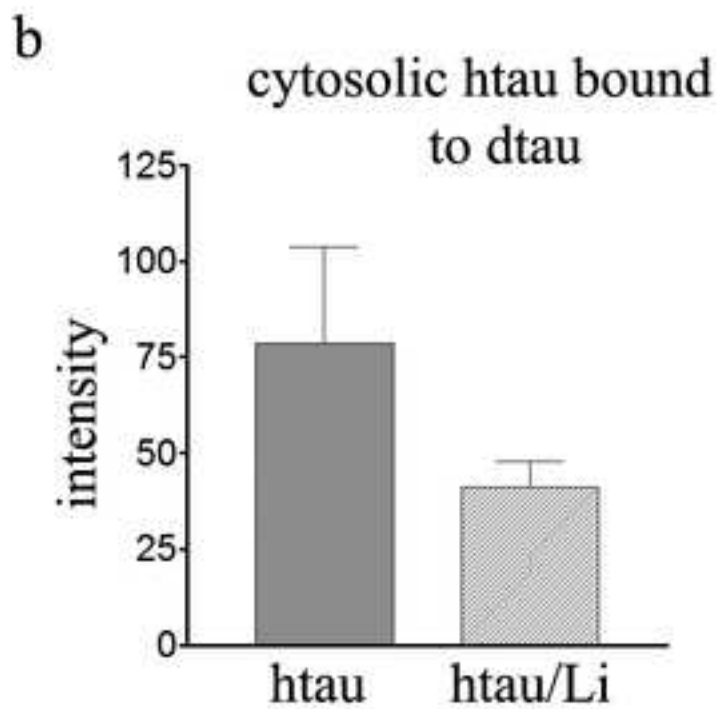
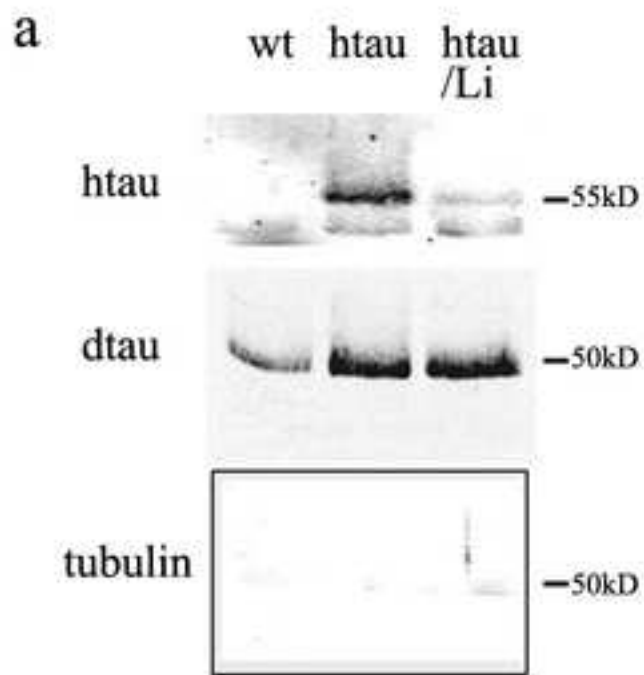


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Fig. 2



## Cowan et al Fig. 3





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