Disruption of neuronal function by soluble hyperphosphorylated tau in a Drosophila model of tauopathy

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Abstract
Axonal microtubules are essential for transport of materials to the synapse. Compromised microtubules and synaptic loss have been demonstrated in AD (Alzheimer’s disease), which is believed to contribute to cognitive dysfunction before neuronal death in the early stages of the disease. The mechanism by which hyperphosphorylated tau, the building block of neurofibrillary tangles, one of the pathological hallmarks of AD, disrupts neuronal and synaptic function is unclear. There is a theory that hyperphosphorylated tau does not bind effectively to microtubules and is no longer able to function in stabilizing them, thus axonal transport can no longer proceed efficiently. This leads to synaptic dysfunction. We have tested this theory in a Drosophila model of tauopathies in which we expressed human tau (h-tau). Using this model, we have tested all aspects of this hypothesis and have demonstrated that axonal transport does become compromised in the presence of hyperphosphorylated h-tau and this leads to synaptic and behavioural defects. We are currently investigating the mechanism by which hyperphosphorylated h-tau mediates this effect and are preliminary data indicate that this entails phospho-tau-mediated effects that are predicted by the tau–microtubule hypothesis, as well as novel effects. These deleterious effects of h-tau occur in the absence of tau filaments and before neuronal death. This sequence of pathogenic events may constitute the mechanism by which abnormal tau disrupts neuronal and synaptic function and contributes to cognitive impairment before neuronal death in the early stages of tauopathies such as AD.

Introduction
Axonal microtubules are essential for transport of materials to the synapse [1]. More than 40 years ago, it was suggested that microtubule-based axonal transport, and therefore synaptic function, might be impaired in AD (Alzheimer’s disease) [2,3]. It has since been shown that reduced numbers of microtubules can be observed in post-mortem AD brain [4]. Similarly evidence implicating synaptic dysfunction in AD comes from the finding that synaptic loss is evident early on in the disease and is closely correlated with clinical symptoms [5].

The stability of microtubules is maintained by the binding of the microtubule-associated protein tau [6]. Although NFTs (neurofibrillary tangles) within neurons were described 100 years ago as a cardinal pathology in AD, it was not until the 1980s that it was appreciated that these structures are composed of tau [7], that tau is hyperphosphorylated in AD [8] and that hyperphosphorylated tau, as in AD, is less able to bind to and stabilize microtubules in vitro [9]. These discoveries led to the formation of the ‘tau–microtubule’ hypothesis of neuronal dysfunction in AD and other tauopathies [10,11] (Figure 1). This states that when tau is hyperphosphorylated, it is less able to bind to microtubules, is not able to stabilize them effectively, causing their breakdown, leading to a reduction in fast axonal transport, and thereby synaptic dysfunction (Figure 1). There are a number of competing hypotheses to explain how abnormally phosphorylated tau might bring about reduced axonal transport, such as by overstabilizing the microtubules and blocking them [12], or via an altered interaction with the motor protein kinesin [13]. Whatever the mechanism, the resulting synaptic dysfunction is thought to contribute to cognitive symptoms before neuronal death, since there is evidence that, in AD, loss of synapses and associated cognitive dysfunction precedes filament and NFT formation [5,14].

A Drosophila model of tauopathy
Although the above tau–microtubule hypothesis describes a potential mechanism by which hyperphosphorylated tau may disrupt neuronal function before NFT formation and neuronal death, it has proven difficult to test in mouse models. This is because of the limitations of rodent models to study aspects of this hypothesis (such as axonal transport) in a truly in vivo manner, and the fact that many of these models exhibit insoluble tau and some neurodegeneration
The tau–microtubule hypothesis predicts that, in tauopathies, (1) tau becomes hyperphosphorylated; (2) this hyperphosphorylation reduces the microtubule-binding ability of the tau and thus detaches from the microtubules; (3) as a result, the microtubules begin to break down; (4) consequently, the axonal transport of vesicles and other organelles to the synapse becomes disrupted, leading to impaired synaptic function; and (5) the hyperphosphorylated tau proteins accumulate in the axoplasm and eventually aggregate into paired helical and straight filaments, ultimately coalescing to form tangles.

Expression of phosphorylated tau causes axonal transport defect and synaptic dysfunction

To study the effect of hyperphosphorylated tau on axonal transport, the h-tau was co-expressed with GFP (green fluorescent protein)-tagged NPY (neuropeptide Y) vesicles in the motor neurons of Drosophila larvae (Figure 2A). As predicted by the tau–microtubule hypothesis, following the expression of h-tau, a profound disruption of axonal transport was evident in these axons [16]. This was characterized by increased retention of the GFP-tagged vesicles along the motor neuron axons, resulting in ‘piling up’ of cargo along the length of the axon (Figures 2B–2D). This axonal transport disruption was severe enough to precipitate locomotor defects in the larvae (Figure 2E) and adults [16]. These effects were dependent upon the phosphorylation state of tau, because increased phosphorylation by co-expression of shaggy (sgg), the Drosophila homologue of the tau kinase GSK-3β (glycogen synthase kinase 3β), exacerbated both the axonal transport and behavioural phenotypes. Conversely, treatment of the tau-expressing larvae with LiCl, which we have shown reduces tau phosphorylation at GSK-3β sites in this system ([16] and C.M. Cowan and A. Mudher, unpublished work), or the GSK-3β inhibitor AR-A014418 inhibited both phenotypes (Figures 2D and 2E). Synaptic function was also impaired at the NMJs (neuromuscular junctions) of these larvae [17]. This was characterized by morphological abnormalities (Figures 2F and 2G), reduced amplitude of excitatory junctional potentials (Figure 2H) and impaired exocytosis of vesicles [17]. Despite these deleterious effects of h-tau on neuronal function, there was no evidence of neuronal death or neurodegeneration, suggesting that they are indicative of early stages of disease. Taken together, these data demonstrate that hyperphosphorylated tau causes neuronal dysfunction by disrupting axonal transport and synaptic function.
Figure 2 | For legend see facing page
Figure 2 | Expression of phosphorylated h-tau causes axon transport and synaptic defects

(A) h-tau and GFP-tagged NPY were expressed in motor neurons of larvae, using the UAS-Gal4 tissue-specific expression system, and axonal transport and synaptic function was studied. Axonal transport was studied by observing the movement of GFP-tagged NPY vesicles in the motor neuron axons of living intact larvae through their transparent cuticles (arrows) (described in [16]). Synaptic function was studied by examining the electrophysiological and morphological properties of the NMJs in opened preparations (arrowheads) (described in [17]). Expression of h-tau results in an axonal transport defect (B and C), as measured by the accumulation of transport vesicles in live larvae (arrows in C, quantified in D), and a locomotion defect, as measured by a righting assay (E) and a battery of other tests [16]. Both phenotypes are rescued by treatment with GSK-3β inhibitors LiCl or AR-A014418 (D and E). The defect in axonal transport is associated with synaptic dysfunction (F-H) [17]. Confocal image projections of wild-type (WT) (F) and h-tau-expressing (G) NMJs on muscle 4 illustrate aberrant bouton structure and size in h-tau-expressing NMJs. Arrows indicate presynaptic 1b boutons; arrowheads indicate abnormally small mini-boutons. (H) Representative samples of evoked excitatory junctional potentials in muscle 4 of wild-type and h-tau-expressing larvae, recorded at 1 Hz after a 50 Hz stimulus, illustrate the reduced synaptic potentials observed at h-tau-expressing NMJs. Reproduced with permission from [16] (B-E) and [17] (F-H).

Figure 3 | Expression of phosphorylated human tau causes a decrease in microtubule number, and reduces the microtubule-binding of endogenous Drosophila tau, in a phosphorylation-dependent manner

Transmission electron microscopy sections of peripheral nerves from third instar larvae expressing h-tau in a single peripheral nerve (outlined in red on the left-hand side in A) lying alongside non-h-tau-expressing peripheral nerve (outlined in blue on the right-hand side in A) illustrating the effect of tau expression on the ultrastructural appearance of the microtubules. In the non-tau-expressing nerve, the axons contain many regularly spaced outlines of intact microtubules (arrowheads in B). In contrast, in tau-expressing axons, there are few such profiles (the intact microtubule profiles evident are highlighted by arrowheads in C). Scale bars, 200 nm.

Expression of phosphorylated tau causes a breakdown of microtubules

We are currently investigating whether, as is predicted by the tau-microtubule hypothesis, the defect in axonal transport that we observed is due to a loss of axonal microtubules. We are using transmission electron microscopy to compare cross-sections of peripheral nerves of tau-expressing larvae with those of control larvae that express the driver alone. Our preliminary data indicate that the expression of highly phosphorylated tau in neurons (outlined in red on the left-hand side in Figure 3A) is associated with a loss of detectable microtubule profiles in axons (arrowheads in Figure 3C). This result is unlikely to be the product of sub-optimal electron microscopy sectioning/fixing processing procedures because a non-h-tau-expressing control peripheral nerve (outlined in blue on the right-hand side in Figure 3A) adjacent to the h-tau-expressing control nerve displays many regularly spaced intact microtubule profiles within the axons (arrowheads in Figure 3B). We are currently investigating this further and trying to unravel the mechanism by which highly phosphorylated tau mediates this effect. We predict that highly phosphorylated h-tau destabilizes the microtubular cytoskeleton in vivo because it exhibits impaired microtubule
Figure 4 | *In vivo* demonstration of the tau–microtubule hypothesis *in vivo*

In our *Drosophila* model of tauopathy, we show that, upon expression of human tau motor neurons (A), (1) h-tau becomes hyperphosphorylated and this leads to a disruption of axonal transport and synaptic function (4) and the ultimate induction of behavioural defects. We predict that this effect emerges because the highly phosphorylated h-tau is unable to bind to microtubules itself (2) and also because it impairs the microtubule-binding capacity of the endogenous *Drosophila* tau (2'). Our preliminary results indicate that this leads to a dramatic reduction in axonal microtubules visible by electron microscopy (3). (B) If these predictions are true, we would propose further that, upon inhibition of tau phosphorylation (1), binding of h-tau and *Drosophila* tau to microtubules is enhanced (2, 2') and microtubule numbers are restored to normal (3). As a result, axonal transport is restored to normal (4). This ultimately leads to a rescue of behavioural phenotype.
binding and also because it diminishes the microtubule-binding capabilities of the normal endogenous Drosophila tau. In so doing, we predict that the highly phosphorylated h-tau triggers a pathogenic cascade within which it converts or ‘poisons’ normal tau and renders it also functionally incompetent (Figure 4). We are currently testing this hypothesis (C.M. Cowan and A. Mudher, unpublished work).

What is the significance of tangle formation in tau-mediated dysfunction?
The tau–microtubule hypothesis goes on further to predict that the tau which has been displaced from the microtubules then forms filaments, which are ultimately the NFTs that are observed in AD and other tauopathies. In various versions of the model, these filaments may themselves be cytotoxic, they may sequester 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. Our work and that of others suggests that filament formation may be a downstream event. There is evidence that, in AD, loss of synapses and associated cognitive dysfunction precedes filament and NFT formation [5,14]. In rodent tauopathy models, animals do eventually acquire NFTs, but long after they exhibit the disease phenotype [21,22]; and in the original Drosophila tauopathy models, fruitflies never develop filaments or NFTs [15,23]. Our studies indicate that tau-mediated neuronal dysfunction is attributable to the phosphorylation state of tau before filament and NFT 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.

Conclusions
We have used a Drosophila model of tauopathy to unravel the mechanisms by which hyperphosphorylated tau causes neuronal dysfunction in vivo (Figure 4A). Hyperphosphorylated tau fails to bind effectively to microtubules, and also prevents normal tau from binding to microtubules, which then allows the microtubules to break down. This results in reduced axonal transport, impaired synaptic structure and function, and thus a behavioural phenotype. Hyperphosphorylation of tau at GSK-3β sites is further required for this pathological process, and we predict further that inhibition of this phosphorylation would restore tau-driven cytoskeleton destabilization and thus enable the maintenance of an efficient axonal transport system (Figure 4B). 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. This work strongly supports the growing opinion that significant neuronal dysfunction precedes overt neuropathological hallmarks.

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