

Tau toxicity is gated through the 306VQIVYK311 domain but through mechanisms not entirely dependent on Tau aggregation

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Tau toxicity is gated through the ³⁰⁶VQIVYK³¹¹ domain but through mechanisms not entirely dependent on Tau aggregation

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Abstract

Disease-modifying therapies for Tauopathies like Alzheimer's disease have targeted Tau hyperphosphorylation and aggregation, as both pathological manifestations are implicated in Tau-mediated toxicity. More recently there has been a renewed interest in Tau conformation which appears to influence its toxic potential. However, the impact of Tau hyperphosphorylation on its eventual conformation and toxic potential is less well known.

Leveraging the genetic tractability of *Drosophila*, we generated multiple inducible human Tau transgenes with altered phosphorylation status and/or aggregation propensity. Their individual and combined impact was tested *in vivo* by quantifying Tau misfolding, accumulation and neurodegeneration in the aging fly nervous system.

Results showed that phospho-mimicking Tau (hTau2N4R^{E14}) induced profound neurodegeneration, supporting a neurotoxic role for phosphorylation. However, deletion of the aggregation-promoting ³⁰⁶VQIVYK³¹¹ motif in the microtubule-binding region altered the conformation of Tau and neurotoxicity was completely abolished. Intriguingly, overt aggregation of Tau into large filaments, detectable as HMW species in biochemical preps, was not significantly reduced. Moreover, a peptide inhibitor targeting this same motif, that we have previously shown to promote off-pathway Tau aggregation, efficaciously reduced Tau-mediated behavioural deficits in aging *Drosophila*.

Collectively we show that neurodegeneration mediated by Tau hyper-phosphorylation is gated via at least one aggregation-promoting motif of the protein, whether through a direct impact on Tau aggregation or through suppression of other mechanisms of toxicity mediated through this domain. Targeting the ³⁰⁶VQIVYK³¹¹ domain reduces the ability of hyperphosphorylated Tau species to acquire the pathological conformation responsible for inducing neurotoxicity. This highlights the primacy of blocking the ³⁰⁶VQIVYK³¹¹ domain of Tau to

alter Tau conformation, in emerging therapeutics, perhaps without the need to clear phosphorylated species.

Introduction

The presence of highly phosphorylated, insoluble inclusions of the microtubule associated protein Tau (MAPT) within neurons and/or glia of the central nervous system (CNS) is a common pathological hallmark of Alzheimer's disease (AD) and other neurodegenerative dementias collectively known as Tauopathies (1-3). Tau hyperphosphorylation and aggregation are the two most prominent pathogenic hallmarks that influence Tau-mediated toxicity and have therefore become the primary targets for modern Tau-based diagnostic biomarkers and therapeutics (4, 5). However, the extent of their interdependence or relative contributions to the progression of Tau-mediated toxicity, neuronal degeneration and disease progression remains unclear.

Physiologically, Tau phosphorylation is implicated in the regulation of Tau-microtubule binding (6), promoting microtubule assembly, stabilisation and spacing of the microtubule network within neurons (7). However, a hallmark of the pathogenic process is Tau becoming increasingly hyperphosphorylated (8), resulting in less efficient binding to microtubules and the induction of toxic consequences at the molecular, subcellular and cellular level. *In vitro* studies have demonstrated that expressing phospho-mimicking Tau in neuronal cells has a profound effect on neuronal structure and function, compromising cytoskeletal integrity (9, 10), suppressing neuronal excitability (11) and inducing significant changes to the normal subcellular localization and distribution of Tau (11-13). Similar deficits are reported *in vivo* in *Drosophila* models of Tauopathy where driving hyperphosphorylation, by co-expression of the Tau kinase GSK-3 β , or emulating hyperphosphorylation by expressing the phospho-mimicking TauE14 transgene disrupted axonal transport (14), reduced microtubule

binding (15) and induced significant neuronal and synaptic degeneration (9, 14, 16-19). In the TauE14 transgene, key disease-associated serines and threonines have been substituted with glutamate, with the charge on the latter mimicking phosphorylation. These manipulations impair neuronal function in multiple ways and induce significant neuronal and synaptic degeneration (9, 14, 16-19). Moreover, expression of TauE14 resulted in greater accumulation of Tau in sarkosyl insoluble cellular fractions and the formation of *bona fide* Tau aggregates within fly brains (17, 20, 21). Furthermore, in FTD mouse models, transgenic Tau variants can misfold and form pre-tangle-like fibrils, which can be significantly reduced with GSK-3 β inhibitor treatment (22-28).

Several species of aggregated Tau have been identified in both human brain and animal Tauopathy models. However, the smaller hyperphosphorylated oligomeric aggregates are thought to mediate toxicity (29, 30). The hypothesis that aggregation of these smaller Tau oligomers mediates Tau toxicity is well-supported in cell culture models, presenting with impaired synaptic function (13), cellular damage (31), disrupted cellular trafficking including axonal transport (32), and induction of inflammatory responses (33). In addition, transgenic animals over-expressing aggregation-prone Tau mutants and isoforms incur significant neuronal degeneration paired with cognitive and locomotor deficits (15, 34-37). Such aggregation-induced toxicity has been attributed to two hexapeptide motifs on the Tau protein, denoted PHF6* (²⁷⁵VQIINK²⁸⁰) and PHF6 (³⁰⁶VQIVYK³¹¹) (37). When these hexapeptides are deleted, or blocked by site-specific peptide-based drugs, cognitive deficits and reduced lifespan are largely ameliorated in transgenic animals (38-40).

Several lines of evidence implicate Tau phosphorylation in promoting aggregation, but the precise molecular mechanism underlying this interplay is not clear. Nonetheless, phospho-epitopes of Tau influence its aggregation, either directly or by promoting phosphorylation at additional sites (17, 41-46). The consensus view is that phosphorylation at residues that reduce

microtubule binding can encourage aggregation by enhancing the local cytosolic concentration of free Tau, thus encouraging interaction with aggregation-promoting cofactors (47). Alternatively, Tau phosphorylation may induce misfolding into seed-competent and aggregation prone conformations (48, 49). Conversely, some studies show that phosphorylation at certain sites appears to suppress further phosphorylation and aggregation (45, 50-53). Complicating the picture further, there is some evidence to suggest that phosphorylation may lie downstream of aggregation. Inhibition of aggregation through ³⁰⁶VQIVYK³¹¹ ablation and peptide-based drugs leads to reduced phosphorylation in *in vitro* and *in vivo* models of Tauopathy (38, 39, 54, 55), suggesting that aggregation may in fact promote phosphorylation. It is therefore unclear whether phosphorylation lies exclusively upstream of aggregation or whether Tau can become phosphorylated to some extent after it is aggregated. Such incongruency may arise from the use of different Tau isoforms in diverse experimental systems which complicate comparisons between different studies addressing the relationship between Tau phosphorylation and aggregation.

Beyond the potential causal and perhaps reciprocal relationship between Tau aggregation and phosphorylation, interpreting the relative contribution of each pathological modification towards Tau-mediated toxicity is challenging. This is because it is difficult, in a single model, to independently and combinatorially manipulate phosphorylation, aggregation, Tau expression levels and neuronal age, all of which contribute to degenerative phenotypes (34, 56, 57). To resolve this conundrum, a systematic assessment of the aggregation-phosphorylation interplay in a well-controlled *in vivo* experimental model is needed.

To address this problem, we assessed the relative contributions of phosphorylation and aggregation on Tau-mediated degeneration using several novel transgenic *Drosophila*. A number of novel human Tau (hTau) mutants were generated to determine toxicity of wild type (WT) and phospho-mimicking Tau (hTau2N4R^{E14}), and mutants with deletion of the

aggregation-promoting ³⁰⁶VQIVYK³¹¹ sequence in both WT Tau (hTau2N4R^{DEL}) and phospho-mimicking Tau (hTau2N4R^{E14/DEL}) to enable the assessment of the toxicity of phosphorylated Tau which cannot aggregate. We report that the expression of WT hTau2N4R induced significant intracellular Tau accumulation and neuronal degeneration, which was greatly exacerbated by the phospho-mimicking hTau2N4R^{E14} variant. However, deletion of the ³⁰⁶VQIVYK³¹¹ sequence completely abolished the toxicity of the phospho-mimicking Tau, demonstrating that whilst hyperphosphorylation of Tau drives Tau-toxicity, this absolutely requires the ³⁰⁶VQIVYK³¹¹ domain. Whether this domain mediates Tau toxicity exclusively by directly promoting aggregation or additionally via indirect mechanisms involving interacting proteins and induction of pathological conformations remains unresolved.

Results

Phosphorylation-dependent somato-dendritic and axonal degeneration requires the ³⁰⁶VQIVYK³¹¹ domain

To investigate the relative contributions of hyperphosphorylation and aggregation to neuronal degeneration, we first generated a panel of 10xUAS-hTau2N4R transgenes with specific mutation(s) and a single copy was integrated into the *attP40* locus of the fly genome. Thus, the mutants were expressed at similar levels in all experiments (Supplementary Fig. 1), allowing quantitative comparison of their impacts. An additional advantage of this system is that all transgenes are selectively expressed in well-defined neurons (Or47b olfactory sensory neurons) offering segregation of neuronal compartments to facilitate assessment of Tau sub-cellular localization and toxic effects in the soma, dendrites, axons and synaptic fields (Supplementary Fig. 2). Or47b neuronal soma and dendrites are in the proximal region of the antennae and their axons innervate and terminate in a specific antennal glomerulus bilaterally, where they synapse with projection neurons relaying olfactory information to higher order learning and memory centres (Supplementary Fig. 2). To identify neurons expressing the

Or47b-GAL4 driver, the membrane tagging *UAS-CD8::EGFP* transgene was recombined onto the same chromosome. Thus, neurons expressing the novel Tau transgenes under this driver were marked by GFP, while Tau expression and its effects were visualized by tracking the cytoplasmic mCherry (i.e. *mCherry::hTau2N4R*).

We generated the following Tau mutant transgenic strains: *UAS-hTau2N4R*, the phospho-mimicking mutant *UAS-mCherry::hTau2N4R^{E14}* in which phosphorylation at 14 disease-associated sites is physiochemically mimicked, the aggregation resistant mutant *UAS-mCherry::hTau2N4R^{ΔVQIVYK}* in which the aggregation-promoting ³⁰⁶VQIVYK³¹¹ motif is deleted (termed hTau2N4R^{DEL}), and also a phospho-mimicking but aggregation resistant mutant *UAS-mCherry::hTau2N4R^{E14/ΔVQIVYK}* (termed hTau2N4R^{E14/DEL}).

Consistent with previous studies, expression of hTau2N4R led to progressive and quantifiable reduction in soma width that was significantly different from controls by day 14 (T14) (Fig. 1A6 *versus* Fig. 1A7, quantified in Fig. 1C). These somato-dendritic defects were exacerbated in animals expressing the phospho-mimicking hTau2N4R^{E14} in which neurodegeneration was significantly enhanced such that there was 23% soma loss by day 3 (T3) which increased to 36% by T14 (Figs. 1A4 and 1A9, quantified in Fig. 1B). Moreover, the width of the soma in hTau2N4R^{E14}-expressing neurons was also significantly reduced by T3 and remained at this level at T14 compared to no Tau and hTau2N4R controls (Figs. 1A9, quantified in Fig. 1C).

In the axonal compartment, Tau accumulation is reported to impair transport(14, 18) resulting in axonal swellings leading to fragmentation and neuronal loss in mammalian Tauopathy models(58-60). These morphological characteristics were also examined in hTau-expressing *Or47b* neurons. Control *Or47b* neurons present with the typical compact highly fasciculated axon bundles with some age-dependent swellings and defasciculation evident in older animals (Fig. 2A1 and Supplementary Fig. 3A1). The number, size and total volume of

axonal swellings were greatly increased upon hTau2N4R expression at all ages assayed (Fig. 2A2 and Supplementary Fig. 3A2). As was seen in the somato-dendritic compartment, these aberrant axonal phenotypes were greatly exacerbated by hTau2N4R^{E14} expression at all time points. This was characterised by the appearance of prominent axonal swellings, fragmentation and dystrophic processes in hTau2N4R^{E14}-expressing neurons (arrow heads in Fig. 2A4 and Supplementary Fig. 3A4), indicative of substantial axonal and synaptic loss. This is also evident in a reduction of the total volume of the axonal bundle (Supplementary Fig. 4).

Both these data sets align with other studies indicating that Tau-mediated somato-dendritic and axonal degeneration is driven by phosphorylation at sites known to be phosphorylated in Tauopathies validating our novel experimental system.

As aggregation is also implicated in Tau-mediated degeneration and is believed to be triggered by misfolding induced by Tau hyperphosphorylation, we next sought to investigate the impact of reducing the aggregation propensity of hTau2N4R and of hTau2N4R^{E14}. This was achieved by deleting the ³⁰⁶VQIVYK³¹¹ domain in both Tau transgenics which are referred to as hTau2N4R^{DEL} and hTau2N4R^{E14/DEL} respectively from here on. Both hTau2N4R-mediated shrinkage of cell body width (Fig. 1A7 and 1C) and axonal swellings (Fig. 2A2) were completely absent upon ³⁰⁶VQIVYK³¹¹ deletion both in hTau2N4R^{DEL} (Fig. 1A8, Fig. 2A3, Supplementary Fig. 3A3), and hTau2N4R^{E14/DEL}-expressing animals at all ages tested (Fig. 2A5, Supplementary Fig. 3A5). The cell body widths and axonal profiles of both these ³⁰⁶VQIVYK³¹¹ deletion mutants were virtually indistinguishable from age-matched controls (Fig. 2A1 and Supplementary Fig. 3A1). Quantification of axonal swellings and neuronal loss further supports these conclusions (Fig. 2B and Supplementary Fig. 4).

Additional confirmation of these findings was afforded by Structured Illumination Microscopy (SIM) which provides much greater subcellular resolution to visualise Tau-mediated axonal degeneration. SIM imaging of the commissural tract located at the top of the

olfactory glomeruli at T14 clearly shows large axonal swellings and profound axonal loss in the hTau2N4R^{E14} animals (Fig. 2A4'), which are completely attenuated upon ³⁰⁶VQIVYK³¹¹ deletion in hTau2N4R^{E14/DEL} animals (Fig. 2A5').

This shows that deletion of the ³⁰⁶VQIVYK³¹¹ motif renders the toxic hTau2N4R protein benign, even in animals mimicking hyperphosphorylation at established disease-associated sites. This implies that the toxicity of hyperphosphorylated hTau2N4R is mediated by the ³⁰⁶VQIVYK³¹¹ aggregation promoting motif.

To test the specificity of this remarkable rescue of phospho-Tau-mediated toxicity by deletion of the ³⁰⁶VQIVYK³¹¹ domain, the same Tau variants were expressed in developing photoreceptors, using a *GMR-GAL4* photoreceptor driver. Tau toxicity in photoreceptors manifests as fusion, loss and disorganisation of ommatidia and sensory bristles (Supplementary Fig. 5), which can be assigned a 'disorganization' phenotypic score using Flynotyper(61). Expression of hTau2N4R led to a subtle eye disorganization (Fig. 2A7), which was significantly exacerbated in the hTau2N4R^{E14} animals (Fig. 2A9). Reminiscent of the rescue seen in the Or47b neurons, these eye phenotypes were also totally eliminated upon deletion of the ³⁰⁶VQIVYK³¹¹ domain in both the hTau2N4R^{DEL} and hTau2N4R^{E14/DEL} animals (Fig. 2A8, A10). Quantification of this data supports this conclusion (Fig. 2C). Collectively, these data indicate that toxicity mediated by hyperphosphorylation of hTau is gated through the aggregation-promoting ³⁰⁶VQIVYK³¹¹ domain in multiple neuronal populations.

Sub-cellular accumulation and mis-localisation of Tau requires the ³⁰⁶VQIVYK³¹¹ domain

Another hallmark of Tau pathology is its mis-localisation or mis-sorting from axonal (62, 63) to the somato-dendritic compartments, where it accumulates in large insoluble cytoplasmic hyperphosphorylated inclusions (13, 62-64). In the Or47b neurons, the hTau is expressed throughout the soma, axons and dendrites (Fig. 3B, Fig. 4A, Supplementary Fig. 6).

However, in the somato-dendritic compartment, hTau2N4R appears to be more highly concentrated within the dendrites that lie within the antennal sensilla (Fig. 3A1-A10), indicating mis-localisation to this compartment. Within the dendrites hTau2N4R distribution became uneven and fragmented from T3 to T14 (Fig. 3A2, A7, Supplementary Fig. 6A2, A7). By comparison, dendritic mis-localisation was greatly exacerbated in an age-dependent manner in hTau2N4R^{E14}-expressing neurons (Fig. 3A4, A9, Supplementary Fig. 6A4, A9). However, deletion of the ³⁰⁶VQIVYK³¹¹ domain either in hTau2N4R or hTau2N4R^{E14} led to a profound reduction of this mis-localisation, as smaller and sparser Tau accumulates were observed within dendrites (Fig. 3A3, A5, A8, A10). This is supported by quantification of the volume of hTau in the dendritic compartment showing significantly greater dendritic accumulation in hTau2N4R^{E14} compared to hTau2N4R, which was significantly reduced in hTau2N4R^{E14/DEL} (Fig. 3C).

In the axonal compartment, all hTau protein variants were uniformly distributed in Or47b expressing axons and synapses at T3 (Supplementary Fig. 3B2-5). However, by T14 the frequency and size of Tau accumulates increased in hTau2N4R-expressing flies consistent with the excessive axonal swellings displayed by these axons (arrowheads in Fig. 4A2). These accumulates almost doubled in Tau2N4R^{E14}-expressing animals (arrowheads Fig. 4A4), relative to those accumulating hTau2N4R (Fig. 4B). These pathological Tau accumulates were suppressed by ³⁰⁶VQIVYK³¹¹ deletion such that by T14, axons were almost devoid of them in hTau2N4R^{E14/DEL} animals and the axonal profiles looked smooth and completely normal (arrowheads in Fig. 4A5 *versus* Fig 4A4). Scrutiny of the axonal bundle using SIM imaging confirms the denser and more prominent intracellular Tau accumulations observed in hTau2N4R^{E14}-expressing flies (Fig. 4A4' *versus* Fig. 4A2') and complete suppression of Tau accumulation in the hTau2N4R^{E14/DEL}-expressing neurons (Fig. 4A5' *versus* Fig 4A4').

Collectively then, the aggregation promoting ³⁰⁶VQIVYK³¹¹ domain drives somato-dendritic mis-localisation and axonal accumulation of hyperphosphorylated Tau.

How does deletion of the ³⁰⁶VQIVYK³¹¹ motif prevent Tau-toxicity?

Our data thus far implies that the pathogenic mechanism employed by hyperphosphorylation of Tau requires the aggregation-promoting ³⁰⁶VQIVYK³¹¹ domain to mediate toxicity. The most parsimonious explanation for the rescue of Tau toxicity following deletion of this domain is a suppression of Tau aggregation. To examine this, a sarkosyl insolubility assay was used to fractionate monomeric, aqueous insoluble but sarkosyl soluble oligomeric and sarkosyl insoluble fibrillar Tau species (65). At day 14, in all the animals, the Tau expressed appears to exist mostly as monomeric and sarkosyl soluble oligomeric forms with some Tau also appearing as fibrillar species (Supplementary Fig. 7A,B for representative blot and 7C,D,E for quantification). To our surprise deletion of the ³⁰⁶VQIVYK³¹¹ domain in hTau2N4R^{DEL} did not alter the proportion of Tau in the sarkosyl soluble oligomeric or fibrillar fractions compared to the hTau2N4R animals (Supplementary Fig. 7A,B). This is despite the fact that there is significantly more neurodegeneration and accumulation of Tau in the Or47b neurons in hTau2N4R animals when compared to the hTau2N4R^{DEL} animals at this time point (for example see Fig 1C and Fig 2B where the toxicity in hTau2N4R animals is significantly greater than that in hTau2N4R^{DEL} animals). In contrast, in the hTau2N4R^{E14} animals, there did appear to be more fibrillar Tau when compared to hTau2N4R animals, which was reduced in the hTau2N4R^{E14/DEL} animals but this was not statistically significant (compare the bands in hTau2N4R^{E14} *versus* hTau2N4R^{E14/DEL} in Supplementary Fig. 7A,B). Overall, this collectively implies that the ³⁰⁶VQIVYK³¹¹ domain may be enhancing Tau toxicity by promoting aggregation only in the phospho-mimicking hTau2N4R^{E14} animals. It gates toxicity through some other mechanism in the hTau2N4R animals. This is an important observation because this domain has always been considered to be pathologically significant only because it is

thought to enable Tau aggregation; our data shows that whilst it may contribute to Tau toxicity through promotion of fibril formation, especially when Tau is hyperphosphorylated, this is not the only way in which it gates Tau toxicity.

To assess how else the ³⁰⁶VQIVYK³¹¹ domain gates Tau toxicity, we examined three other potential pathogenic mechanisms: i) alteration of phosphorylation capacity, ii) alteration of microtubule binding ability and iii) propensity to misfold and acquire pathogenic conformations.

As Tau toxicity is clearly driven by its phosphorylation state, and some studies have reported a direct relationship between aggregation and phosphorylation, such that the former impacts the latter (38, 39, 54), we investigated the effect of deleting the aggregation promoting ³⁰⁶VQIVYK³¹¹ domain on subsequent downstream phosphorylation of Tau. We used western blot analysis to examine phosphorylated Tau (p-Tau) expression with classic antibodies AT8, AT180, AT270, PHF-1, and Ser262, which detect p-Tau at AD-relevant epitopes (Supplementary Fig. 8). Since TauE14 is mutated at most of these sites, antibody binding would not occur, so we conducted the analysis in WT hTau2N4R and hTau2N4R^{DEL}. After 14 days of expression there was no significant difference in total Tau expression (Supplementary Fig. 8A) which implies the ³⁰⁶VQIVYK³¹¹ domain did not alter the clearance of Tau. Moreover, the phosphorylation of Tau at all epitopes tested; Ser202, Thr205, Thr231, Thr181, Ser396, Ser404 and Ser262 were unchanged by the ³⁰⁶VQIVYK³¹¹ deletion (Supplementary Fig. 8A, A'). This implies that deletion of the aggregation-promoting ³⁰⁶VQIVYK³¹¹ domain does not change the ability of Tau to be phosphorylated. Thus the reduced toxicity of hTau2N4R^{DEL} compared to hTau2N4R is not because of any change in phosphorylation status.

The ³⁰⁶VQIVYK³¹¹ domain is located in one of the four microtubule binding domains of Tau, and though not directly thought to be in the microtubule-binding interface, it is believed

to play a role in regulating microtubule binding function (66). It is conceivable that deletion of this domain renders the hTau2N4R^{DEL} and hTau2N4R^{E14/DEL} proteins functionally incompetent, so their expression in the cells is inconsequential and does not cause any toxic gain or loss of function effect. To test for this a simple microtubule binding assay was carried out and as shown in Supplementary Fig. 9, the microtubule binding ability of all the Tau variants used in this study are not different. These findings demonstrate that the ³⁰⁶VQIVYK³¹¹ deletion variants are not biologically inert or nonfunctional proteins. Rather, they are expressed and retain measurable cellular activity, indicating that the absence of toxicity cannot be attributed to a trivial loss of function or failure to engage cellular pathways.

For over a decade there have been many studies showing that soluble Tau oligomers, likely forming early on in the disease process (67), play a key role in Tau toxicity, in some cases even more prominently than insoluble fibrils (68, 69). Misfolding to adopt a pathogenic conformation is an early step in the formation of such soluble oligomers and the MC1 antibody has been used extensively to detect such misfolded Tau species in both human postmortem tissue as well as in rodent models of Tauopathy (70-76). To test whether the mechanism by which deletion of the ³⁰⁶VQIVYK³¹¹ domain conferred protection was through reduced pathogenic misfolding, potentially suppressing formation of toxic soluble Tau oligomers, the MC1 antibody was used in an immunohistochemical analysis. As depicted in Supplementary Fig. 10, MC1 staining was specific to Tau-expressing flies, with no signal detected in no Tau controls. After 14 days of aging, all the Tau expressing flies expressing exhibited some MC1-specific labelling, restricted to small puncta ~0.2-2µm in size within the Or47b neurons (Supplementary Figure 10. B3, C3, and E3). MC1 positive accumulates in these animals colocalised strongly with mCherry::Tau and were primarily localised to the axonal terminals at the periphery of the Or47b glomerulus, representing only a minor proportion of the total Tau signal (Supplementary Figure 10. B2 *versus* B3, C2 *versus* C3, and E2 *versus* E3). MC1

immunoreactivity was significantly increased in the hTau2N4R^{E14} animals when compared to hTau2N4R (Supplementary Figure 10. B3 versus D3). Deletion of ³⁰⁶VQIVYK³¹¹ domain significantly reduced this MC1 phenotype in the hTau2N4R^{E14/DEL} animals (Supplementary Figure 10. D3 *versus* E3, quantified in Supplementary Figure 10. B). There was a similar trend for reduction of MC1 immunoreactivity in hTau2N4R^{DEL} animals when compared to hTau2N4R but the effect was small and not statistically significant (Supplementary Figure 10.B). This data implies that the ³⁰⁶VQIVYK³¹¹ domain confers protection against the toxicity of hyper-phosphorylated Tau by suppressing its ability to misfold into a pathogenic conformation, possibly preventing formation of toxic soluble Tau oligomers.

Peptide inhibition of ³⁰⁶VQIVYK³¹¹ rescues Tau-mediated locomotor deficits

To explore the translational potential of targeting the ³⁰⁶VQIVYK³¹¹ domain, we tested whether targeting it pharmacologically could suppress pathological manifestations of hTau. To that end, flies expressing hTau2N4R pan-neuronally were fed a low-dose (0.08μM referred to as LD) and high-dose (0.8μM referred to as HD) of an aggregation inhibitor peptide RI-AG03. Designed especially against the ³⁰⁶VQIVYK³¹¹ domain, we have previously shown that RI-AG03 effectively suppresses aggregation of hTau *in vitro* in a biosensor cellular model (40) . This inhibitor is well tolerated and not toxic to *Drosophila* at the LD and HD doses we have used in the present study (40). We studied the climbing ability of adult flies in a well-established locomotor assay to assess the effects of hTau expression in aging flies (34, 38). We quantified the height climbed by flies in a 10 second period and found that the climbing ability of control flies declined progressively, by 30% over 35 days (Fig. 4C). In contrast, expression of hTau2N4R reduced climbing efficiency by 40% at T1 and by 70% at T21 when compared to age-matched controls (Fig. 4C). By T35, hTau2N4R-expressing flies were unable to climb at

all. However, treatment with either the LD or HD of RI-AG03 significantly improved the climbing ability of hTau2N4R-expressing flies, at every time point tested, though not to control levels (Fig. 4C). There did not appear to be any greater improvement in the flies treated with the HD compared to those treated with the LD of RI-AG03. Although the peptide targets and inhibits the ³⁰⁶VQIVYK³¹¹ domain, its effects are unlikely to be as potent as the complete rescue of toxicity we reported when this motif is deleted in hTau2N4R^{DEL}-expressing flies (Fig. 1-4), as drug efficacy is highly dependent on its stability, penetration, assimilation and pharmacokinetics. Nonetheless, this data collectively suggests that pharmacological inhibition of the ³⁰⁶VQIVYK³¹¹ domain is an effective method to suppress aggregation-dependent Tau phenotypes.

Discussion

Phosphorylation and aggregation are both established pathogenic post translational modifications of Tau thought to play a central role in mediating Tau-toxicity. They therefore represent significant disease-modifying therapeutic targets for the treatment of Tauopathies. Yet the causal relationship between these distinct post-translational modifications and their relative contribution towards Tau toxicity is not clear. Our study determines the relative contribution of hyperphosphorylation and aggregation in Tau-mediated neurodegeneration in aging *Drosophila* olfactory neurons. We show that ³⁰⁶VQIVYK³¹¹ is an excellent candidate for therapeutic intervention *in vivo*, and provide novel data which shows that its targeting can render even highly phosphorylated Tau species inert. Our data also indicates that this domain is not necessarily gating toxicity simply by promoting aggregation of Tau into insoluble species like fibrils. It is likely to also mediate toxicity through other, less understood mechanisms, that begin with induction of a misfolded conformation.

375

376 **Phosphorylation as a Key Driver of Tau-mediated Toxicity**

377 We demonstrate that the pseudo-phosphorylated hTau2N4R^{E14} mutant significantly
378 exacerbates neurodegenerative phenotypes compared to hTau2N4R in various neuronal
379 compartments in a novel experimental system. This finding aligns with previous research using
380 TauE14 and other phospho-mimicking mutants which have consistently shown increased
381 neuronal loss and organizational defects when expressed in alternative *Drosophila* neuron
382 subtypes including the mushroom bodies (77), retinal neurons (20, 78), and circadian clock
383 neurons (79). However, focusing on Or47b neurons revealed not only severe morphological
384 deficits, such as profound loss of somatic and axonal tissue, but also subtler hallmark
385 manifestations more difficult to document *in situ* such as the marked increase in axonal
386 swelling size and frequency.

387 While TauE14 is an aggressive mutant mimicking extensive hyperphosphorylation akin
388 to that seen in AD, it effectively recapitulates the pathogenesis associated with pathological
389 Tau hyperphosphorylation. This underscores the causal role of the 14 GSK-3 β phosphorylation
390 sites mutated in TauE14, in Tau-mediated toxicity and validates the focus on reducing
391 phosphorylation as a therapeutic strategy. Adding to this we now show, for the first time, that
392 the pathogenic potential of these sites requires the aggregation promoting ³⁰⁶VQIVYK³¹¹
393 domain.

394

395 **The Critical Role of the ³⁰⁶VQIVYK³¹¹ Domain in Tau Toxicity**

396 There is significant evidence suggesting that phosphorylation is a precursor for
397 aggregation. Using pseudo-phosphorylated Tau mutants, like TauE14, multiple groups have
398 identified a number of phosphorylation sites that act in concert to promote polymerization of

monomeric Tau (44-46, 51, 80-82). Other studies using TauE14 have shown increased levels of Tau in sarkosyl-insoluble fraction in *Drosophila* suggestive of increased hTau aggregation in these pseudo-mutants (20, 78).

Our data largely support these studies, and show that toxicity driven by hyperphosphorylation is fundamentally dependent on the aggregation-promoting ³⁰⁶VQIVYK³¹¹ domain. Remarkably, deletion of this domain in both hTau2N4R and hTau2N4R^{E14} almost completely eliminated toxicity, despite persistent presence of phospho-mimicking epitopes at disease-associated sites. Consistent with our findings, a previous study similarly showed that deleting the ³⁰⁶VQIVYK³¹¹ motif in a *Drosophila* model expressing hTau0N4R attenuated Tau toxicity, rescuing retinal degeneration and shortened lifespan (38).

However, our data additionally suggests that the ³⁰⁶VQIVYK³¹¹ motif does not simply promote aggregation of Tau; it likely mediates toxicity through additional, less studied mechanisms, that begin with induction of misfolding and acquisition of a pathogenic MC1 positive conformation. Many physiological functions of Tau are highly dependent upon its phosphorylation state and its conformation, including interactions with other cellular proteins like RNA-binding proteins, molecular chaperones, signal and membrane-associated proteins (83). Alterations to Tau's global conformation mediated by mutagenic phospho-mimicking mutations and/or deletions may very well shift the mutants interactome profile which may directly or indirectly compromise binding of protective RNA-binding proteins and chaperones such as G3BP2, Hsp27/70/90 and FKBP, which directly bind to the ³⁰⁶VQIVYK³¹¹ motif (84-87).

Irrespective of the mechanism by which deletion of the ³⁰⁶VQIVYK³¹¹ motif protects against Tau toxicity, our data highlights the therapeutic potential of targeting this domain *in vivo*. Building on this, we provide novel insight into its critical role in the context of disease-

related hyperphosphorylation, suggesting that the gain-of-function (GOF) toxicity driven by ³⁰⁶VQIVYK³¹¹-dependent mechanisms surpasses any phosphorylation-mediated contributions to overall Tau toxicity.

Implications for Therapeutic Strategies

Disease-modifying treatments for Tauopathies invariably aim to reduce pathogenic Tau species, whether that is through anti-sense oligonucleotide (88) or vaccination (89) mediated clearance, reduction of Tau phosphorylation through kinase inhibition (90-92) or suppression of Tau aggregation using aggregation inhibitors (39, 40, 55, 93, 94).

Our findings have significant implications for the design of Tau-centred disease-modifying therapeutics. While reducing phosphorylation remains a valid approach, our results suggest that targeting the ³⁰⁶VQIVYK³¹¹ domain may be a more effective strategy to suppress the toxicity of multiple pathogenic forms of Tau. This domain appears to be crucial for pathogenic mechanisms downstream of hyperphosphorylation, beyond simply promoting aggregation, in mediating the toxic effects of Tau. Indeed, several studies have reported promising findings showing the neuroprotective effect of peptides targeting both the ³⁰⁶VQIVYK³¹¹ and the ²⁷⁵VQIINK²⁸⁰ domain. We also show that use of the peptide aggregation inhibitor RI-AG03 (40) to pharmacologically recapitulate the deletion of the ³⁰⁶VQIVYK³¹¹ domain, reduces age-related Tau-mediated behavioural deficits.

Further studies should investigate other physiological and pathological functions of the ³⁰⁶VQIVYK³¹¹ domain including its interactome profile to understand how the deletion of this motif confers protection. Additionally, exploring the effects of peptide aggregation inhibitors on other neurobiological functions of Tau will be crucial for translating these findings into effective therapeutic interventions.

Conclusion

In conclusion, our study provides compelling evidence that while Tau phosphorylation is a key driver of toxicity, its pathogenic effects are primarily mediated through the ³⁰⁶VQIVYK³¹¹ domain. Though traditionally thought to promote aggregation, this domain may mediate Tau toxicity through other, as yet well understood mechanisms as well. Importantly, regardless of the level of Tau phosphorylation, targeting of this domain can effectively suppress Tau toxicity *in vivo*.

Figure Titles and Legends

Figure 1. Phosphorylation dependent somato-dendritic degeneration requires aggregation.

(A) Maximum projection confocal images of the adult antenna of flies expressing membrane bound GFP (CD8::GFP) at the Or47b neurons. Columns represent genotypes expressing different mCherry-tagged hTau2N4R variants plus the driver alone expressing GFP only as a control (left most column). Rows represent post-developmental age (3 or 14 days). Magnified projections (30 slice depth) of the soma region are included. Asterisk shows the reduced soma density induced by hTau2N4R^{E14} expression (A9), which is rescued in hTau2N4R^{E14/DEL} (A10).

(B-C) Quantification of cell bodies per antenna (B) and mean cell body width (C) for the indicated genotypes and time points. Cell bodies were manually counted and measured using ImageJ. Graphs represent mean ±SD, and dots represent averages per antenna. * = p<0.05 (2-way ANOVA with Tukey's multiple comparisons). N= 6-12 antennas collected from two biological repeats per genotype.

Figure 2. Phosphorylation dependent axonal and synaptic degeneration requires aggregation.

(A) Maximum projection confocal (A1-5) or SIM (A1'-5') images of the brain and antennal lobe of flies expressing membrane bound GFP (CD8::GFP) at the Or47b neurons enhanced with α-GFP. Columns represent genotypes expressing different mCherry-tagged hTau2N4R variants plus the driver expressing GFP only as a control (left most column). Rows represent post-developmental age 14 days. Normal structure of the neurons shows some axonal swellings (Arrows) that occur with age and are increased in WT hTau2N4R flies (A2) and exacerbated in hTau2N4R^{E14} expressing flies (A4). Green inset box on A1 highlights the Or47b glomeruli commissure, imaged with SIM (A1'-5') in the middle row.

Bottom row of images show SEM micrographs (A6-A10) of day 14 *Drosophila* eyes expressing hTau2N4R mutants using the GAL4-ninaE.GMR driver (at 1600x magnification). Abnormal retina and degenerated cells are labelled with arrowheads.

(B) Quantification of axonal swellings were automatically quantified using 3D images with IMARIS (see methods). Graphs represent mean ±SD, and dots represent individual values. * = p<0.05 (2-way ANOVA with Tukey's multiple comparisons). N= 6-14 brains collected from two biological repeats per genotype.

(C) Quantification of retinal degeneration using a phenotypic scoring system Flyntyper plugin. Graphs represent mean $\pm$ SD, and dots represent individual values. * = $p < 0.05$ (2-way ANOVA with Tukey's multiple comparisons). N=6 per genotype.

Figure 3. Phosphorylation dependent dendritic Tau accumulation requires aggregation.

(A) Midline 30 slice stacked confocal images of the adult antenna of flies labelled by the mCherryTau signal from expressed mutants in the Or47b neurons. Columns represent genotypes expressing different mCherry-tagged hTau2N4R variants plus the driver expressing GFP only as a control (left most column). Rows represent post-developmental age (3 or 14 days). Magnified regions of interest over the dendrites are presented (A1-A10).

(B) Example image of whole mount antenna, of A2. Arrows point towards Tau expression present within the dendrites, somas and axons. Other whole mounts can be viewed in supplementary figure 6.

(C) Quantification of the volume of dendritic Tau in the antenna was quantified using 3D projection images with IMARIS with a surfacing tool using N=6-12 from two biological repeats. Graphs represent Mean $\pm$ SD. * = $p < 0.05$ (2-way ANOVA with Tukey's multiple comparisons).

Figure 4. Phosphorylation dependent axonal and synaptic Tau accumulation requires aggregation, and treatment of aggregation inhibitor RI-AG03 rescues locomotive dysfunction in Tau-expressing *Drosophila*.

(A) Maximum projection confocal (A1-5) or SIM (A1'-5') images of the antennal lobe in the brain of day 14 old flies expressing mCherry::Tau mutants in the Or47b neurons (enhanced with α -hTau Dako). Arrows point toward beaded Tau accumulates within expressing neurons which increase in frequency and size with age and are exacerbated in hTau2N4R^{E14} expressing flies (A4), but are significantly suppressed in hTau2N4R^{DEL} (A3) and hTau2N4R^{E14/DEL} (A5). Red inset box on A2 highlights the Or47b glomeruli commissure, imaged with SIM (A1'-A5') at T14 on the bottom row.

(B) Quantification of the Tau accumulate density were quantified using 3D images with IMARIS with a spotting tool (see methods) N=6-14 from two biological repeats. Graphs represent mean $\pm$ SD. * = $p < 0.05$ (2-way ANOVA with Tukey's multiple comparisons).

(C) Climbing assay was used to assess locomotive function of pan-neuronal hTau2N4R expressing flies treated with RI-AG03. Bar graph showing the average height climbed by each group (5 cohorts of 10 male flies) over a 35 day period. Control group (ELAV;OreR) and hTau2N4R-expressing flies were subjected to low-dose (0.08uM) (LD) and high-dose (0.8uM) (HD) treatments of the aggregation inhibitor RI-AG03 from 1 to 35 days of age. hTau2N4R-expressing flies exhibited a significant reduction in normal climbing activity compared to control flies. Treatment with RI-AG03 at both LD and HD significantly improved the climbing performance of hTau2N4R-expressing flies. Error bars represent mean $\pm$ SEM. * = $p < 0.0001$ (2-way ANOVA with Tukey's multiple comparisons).

Supplementary Figure 1. Tau is equally expressed by all mutants.

(A) Western immunoblot analysis of total Tau (α -hTau Dako), taken from 15 male, 2 day old homogenised transgenic fly heads expressing wildtype mCherry::hTau2N4R and mutant forms. Driver is GMR.ninaE-GAL4. Representative blot is shown (A) and mean quantification plotted as a bar graph (A') (n=3 biological repeats). Error bars presented as means with SEM. β -actin was used as the loading control. mCherry::hTau constructs each run at ~100kDa due to the extra weight of the fluorescent tag (hTau2N4R usually runs at ~70kDa) and Actin runs between 35 and 55kDa, at ~45kDa.

Supplementary Figure 2. The Or47b expressing olfactory sensory neurons.

Panel A. Schematic panel orientation of the adult *Drosophila* brain, showing the location of the olfactory receptor 47b (Or47b) neurons expressing membrane bound GFP (green), co-expressing mCherry tagged human Tau2N4R (mCh::hTau) (magenta). Representative confocal images of whole mount (A) Antenna and (C) the Antennal lobe (AL) is shown. (B) Schematic diagram of the Or47b olfactory neurons. The AL is outlined in dashed black, containing multiple olfactory glomeruli, including Or47b (green) containing a bundle of ~60 olfactory receptor neurons (ORNs). ORN cell bodies are contained within the 3rd antennal segment of the Antenna (A, A') and its dendrite projects into the sensilla hairs of the antenna. The axons project from the antenna connecting into the (C) AL of the brain, containing the (C') axonal bundles and synaptic terminals of the Or47b neuron. For simplicity, only a few of the 60 ORNs are shown.

Supplementary Figure 3. T3 Axonal degeneration and Tau accumulation.

(A) Maximum projection confocal images of the adult antenna of flies expressing membrane bound GFP (CD8::GFP) at the Or47b neurons at day 3. Columns represent genotypes expressing different mCherry-tagged hTau2N4R variants plus the driver GFP only control (left most column). (B) Maximum projection confocal (B1-5) images of the brain, antennal lobe of day 3 old flies expressing mCherry::Tau mutants in the Or47b neurons (enhanced with α -hTau Dako). Arrows point toward beaded Tau accumulates.

Supplementary Figure 4. Total volume of GFP expressing neurons.

Quantification of neuron volume was automatically quantified using 3D images with IMARIS (see methods). Graphs represent mean $\pm$ SD, and dots represent individual values. * = $p < 0.05$ (2-way ANOVA with Tukey's multiple comparisons). N= 6-14 brains collected from two biological repeats per genotype.

Supplementary Figure 5. *Drosophila* eye diagram, phosphorylation dependent retinal degeneration requires aggregation.

(A) SEM micrograph of whole *Drosophila* head. White insert shows the posterior region of interest where Tau-mediated retinal degeneration occurs using the GAL4-ninaE.GMR driver. (A') The *Drosophila* eye comprises of ~800 single ommatidia units (highlighted in yellow) which are morphologically sensitive to toxic proteins like Tau. Loss of sensory bristles (blue arrows) are also markers of retinal health, which are viewed using SEM techniques. (B) Digital microscope images (600x) of *Drosophila* eyes expressing each hTau2N4R mutant. A rough appearance of the posterior half of the eye appears after hTau2N4R expression (B2) and is exacerbated by the TauE14 mutations (B4).

Supplementary Figure 6. T3 and T14 somato-dendritic Tau accumulation in whole mount antenna.

Midline 30 slice stacked confocal images of the adult antenna of flies labelled by the mCherryTau signal from expressed mutants in the Or47b neurons. Columns represent genotypes expressing different mCherry-tagged hTau2N4R variants plus the driver GFP only control (left most column). Rows represent post-developmental age 3 or 14 days.

Supplementary Figure 7. Aqueous soluble, sarkosyl-soluble and sarkosyl-insoluble fractions isolated from transgenic *Drosophila* brain homogenates.

Comparison of aqueous soluble monomeric Tau (S1), sarkosyl soluble, oligomeric Tau (S2) and sarkosyl insoluble, fibrillar Tau(S3) and enriched fractions between different transgenic flies tested after 14 days of aging. Driver used was GAL4-GMR.ninaE. Genetic crosses set up at 25°C and progeny aged at 29°C (n=164 heads 50:50 male:female). (A-B) Representative Western blots probed with anti-hTau (Dako) comparing aqueous soluble fraction to A) sarkosyl insoluble and B) sarkosyl soluble fractions. mCherry-tagged Tau constructs migrate at ~100 kDa. (C-D) Quantitative analysis of fraction ratios normalised to respective inputs: C) sarkosyl insoluble:aqueous soluble Tau and D) sarkosyl insoluble:sarkosyl soluble Tau and E) The relative distribution of all Tau fractions. No significant

differences were observed between genotypes in any fraction or ratio analysed. Graphs represent mean $\pm$ SD (N=3 biological replicates). Statistical analysis performed was a one-way ANOVA.

Supplementary Figure 8. Aggregation inhibition does not alter p-Tau expression at multiple Alzheimer's disease relevant sites in aged hTau2N4R expressing *Drosophila*.

Western immunoblot analysis and quantification of (A) total Tau (a-hTau Dako) and p-Tau expression at AT8 (Ser202,Thr205), AT180 (Thr231), AT270 (Thr181), PHF-1(Ser396, Ser404) and Ser262 epitopes did not significantly change between 14 day old transgenic flies expressing WT hTau2N4R and hTau2N4R^{DEL}. Error bars presented as means $\pm$ SEM. Driver is GMR.ninaE-GAL4. (A') Representative blot is shown. β -actin was used as the loading control. mCherry::hTau2N4R constructs each run around 100kDa due to the extra weight of the fluorescent tag and actin runs at ~45kDa

Supplementary Figure 9. All mutants bind to microtubules to the same extent.

A) Representative western blot and B) quantitative analysis of microtubule (MT)-bound Tau in 14-day-old male transgenic flies expressing Tau in adult retinal neurons using the GAL4-ninaE.GMR driver. Brain homogenates were fractioned into microtubule-bound (pellet) and unbound, free Tau (supernatant). mCherry::Tau constructs migrate to ~100kDa and α -Tubulin (loading control) migrates to ~50kDa. Error bars are plotted $\pm$ SD (N=3 biological repeats.). Statistical analysis was performed using a one-way ANOVA, and no statistical significance was reported between genotypes when *p<0.05.

Supplementary Figure 10. ³⁰⁶VQIVYK³¹¹ deletion rescues phosphorylation dependent production of pathogenic Tau conformations.

A) Maximum projection confocal images of the antennal lobe in the brain of 14-day-old flies expressing mCherry::Tau mutants in the Or47b neurons. Rows represent isolated channels 1) membrane bound CD8::GFP tagging the neuron morphology, 2) mCherry::Tau and 3)MC1 antibody detecting pathological misfolded conformations of Tau and 4) composite image of mCherry::Tau and MC1 to show the co-localisation of misfolded Tau within the total Tau expressed. Arrows indicate MC1-positive accumulations, which are substantially more abundant and extensive in hTau2N4R^{E14} neurons compared to all other genotypes. B) Quantification of misfolded Tau proportion within expressing neurons using MATLAB-based automated segmentation of GFP signal to distinguish neuron-specific (GFP-positive) from background (GFP-negative) pixels. Fluorescence values represent average MC1 signal within GFP-positive pixels minus background signal from GFP-negative pixels. hTau2N4R^{E14} shows significantly elevated misfolded Tau levels, which are rescued by ³⁰⁶VQIVYK³¹¹ deletion in hTau2N4R^{E14/DEL}. Graphs represent mean $\pm$ SD (N=4 from single biological replicate). Statistical analysis: one-way ANOVA with Tukey's multiple comparisons (***p<0.001, ****p<0.0001).

Supplementary Figure 11. Using IMARIS software for quantification of Tau accumulation and neuronal degeneration using “spots” and “surfaces” masking tools.

Methods and materials

Fly stocks. *Drosophila melanogaster* were grown on standard cornmeal media in a controlled 12/12 hour light/dark cycle at constant humidity. Each genetic cross was set at 18°C, and selected progeny was collected and moved to 29°C to age for 3 or 14 days. The UAS/GAL4 binary expression system was used for human Tau expression(95) (95), with drivers Or47b-GAL4 and ninaE.GMR-GAL4.

Transgenic fly lines.

GAL4 driver lines. Olfactory driver *+/+* Or47b-GAL4,UAS-mCD8::GFP/Cyo;*+/+* for expression of Tau variants in a small bundle of olfactory sensory neurons (Odorant receptor 47b – VA1d glomeruli) was used for confocal and structured illumination microscopy. Retinal driver *+/+*; GAL4-ninaE.GMR/Cyo; *+/+* for strong expression of Tau variants in photoreceptor cells, utilised for western blot immunoassays.

Tau lines. All UAS-Tau lines (UAS-mCherry::hTau2N4R, UAS-mCherry::hTau2N4R^{Δ306VQIVYK311}, UAS-mCherry::hTau2N4R^{E14} and UAS-mCherry::hTau2N4R^{E14-Δ306VQIVYK311}) were created by the Allan Lab (University of British Columbia, Canada). Tau E14 is generated by mutating 14 AD-relevant epitopes Thr111, Thr153, Thr175, Thr181, Ser199, Ser202, Thr205, Thr212, Thr217, Thr231, Ser235, Ser396, Ser404 and Ser422 to glutamate(13). All UAS-Tau variants were integrated into the same attp40 site on the second chromosome, ensuring the same expression of each Tau variant to allow for direct comparison of toxicity (Supplementary Figure 2).

Confocal imaging experimental procedure

645 ***Tissue preparation***

646 ***Drosophila brains.*** This study utilised the *Or47b*-GAL4 driver. Adults were collected 0-1 days
647 after eclosion and moved from 18°C → 29°C to be aged for either 3 or 14 days. Flies were
648 anaesthetised with CO₂ and the males were selected for dissection. Heads were removed and
649 fixed for 20 minutes in 4% paraformaldehyde (PFA) in phosphate-buffered-saline with 0.2%
650 Triton-X 100 (PBST). The heads were then washed 3 times, for 20 minutes each in PBST, and
651 then dissected under a light microscope in PBST. Brains were then blocked in 3% Normal Goat
652 Serum in PBST (LAMPIRE Biological Laboratories) for 1 hour. Then, primary antibodies were
653 added and incubated overnight. The following day, the primary antibodies were removed and
654 the brains were washed 3 times, for 10 minutes each in PBST. The brains are then left to
655 incubate with the secondary antibodies for 2 hours. Before mounting, the brains are again
656 washed 3 times, for 10 minutes each in PBST. Primary antibodies used: chicken-Anti-GFP
657 (1:1000)(Abcam ab13970), α -human Tau (1:1000) (rabbit-Anti-human-Tau Dako),
658 MC1(1:200). Secondary antibodies used: Goat-Anti-Rabbit-Alexa-555 (1:500) (Abcam
659 Ab150078), Anti-Mouse-Alexa-488 (1:500) (Invitrogen A11039). ***For confocal imaging*** the
660 brains are then transferred to a poly-L-Lysine coated slide containing coverslip well, and
661 mounted using Vectashield® hard-set mounting media (H-1400-10). Slides were stored in a
662 freezer (-20°C) and imaged within 3 days. ***For axonal and synaptic structured illumination***
663 ***microscopy (SIM) imaging***, after antibody incubation brains were post-fixed in 4% PFA for 10
664 minutes, washed and mounted on Superfrost plus adhesion glass slides. Prolong Gold™ is
665 placed on top of the brains and a high-precision thin coverslip (18x18 mm 170± 5µm gently
666 layered on top. Slides left to cure for 24 hours (at room temperature in the dark) before being
667 imaged.

668 ***Drosophila antenna.*** The same progeny was used for antennal dissection. Only one antenna
669 was taken from each animal to avoid the same repeats from the same animal. One antenna was

670 removed from each animal and fixed for 20 minutes in 4% PFA with 0.2% PBST followed by
671 3 PBST washes and immediate mounting onto a poly-L-Lysine coated slide using Vectashield
672 hard-set mounting media. No antibodies were used because the adult antennal cuticle is too
673 thick and highly autofluorescent, instead the endogenous mCherry and CD8::GFP fluorophores
674 were captured during imaging.

675 ***SEM, Drosophila eye.*** 14 day old flies were euthanised, fixed in 4% formaldehyde 3%
676 glutaraldehyde in 0.1M PIPES buffer. Samples were rinsed in 0.1M PIPES buffer twice,
677 immersed in 2% osmium tetroxide for 1 hour, then dehydrated for 20-minutes at each ethanol
678 concentration: 30%, 50%, 70%, 80%, 90%, 100%, 100%. Samples were critical point dried,
679 mounted on aluminium stubs before sputter coating with gold/palladium in a Polaron E5100
680 sputter coater. Samples were examined using a Quanta 250 FEG-SEM.

681

682 ***Image acquisition***

683 ***Confocal Image acquisition.*** Images of the entire *Drosophila* brain and antenna were taken
684 with a Leica SP8 line scanning confocal microscope. Images were taken with 12-bit depth
685 stacks at 63x magnification in glycerol immersion oil, taken at 1024x1024 resolution. 488nm
686 laser was used to detect degeneration of the Or47b neurons and 555nm laser to detect Tau
687 signal. Two sequential excitation steps are used to avoid bleed through between channels.
688 Representative images shown are presented as full Z-stacks, unless otherwise stated. As only
689 structural parameters were evaluated, confocal acquisition settings were adjusted if necessary
690 to capture all structural information.

691 ***SIM image acquisition*** Commissure tract of the Or47b axons within the brain were imaged via
692 SIM (DeltaVision OMX Flex super-resolution microscope) with a 63x Immersion oil objective.

An immersion oil with a refractive index of 1.510 (Cargille Laboratories USA) was used whilst objective collar settings were adjusted to 013 to provide high-resolution images.

Image and statistical analysis

Quantitative analysis of axonal swelling density, Tau accumulate density, volume of dendritic Tau per cell and total volume of GFP expressing neurons were performed using the IMARIS (Supplementary Fig.11) 3D image analysis software. Quantification of cell body density and cell body widths was performed in ImageJ using the cell counter tool and measuring tool respectively. Only one antenna was imaged from one fly. Both hemispheres of the brain were analysed, however in some instances where only one hemisphere was available, quantification was doubled.

Quantification was always carried out using defined parameters. To quantify the number of axonal swellings, the IMARIS “spotting” tool was used to discretely measure the number of axonal swelling surfaces of $\geq 1.2 \mu\text{m}$ with a surface detail of $0.36 \mu\text{m}$. Axonal swelling density was then calculated using the total volume of GFP expressing neurons, which was measured using the “surfacing” tool detecting structures $\geq 0.5 \mu\text{m}$ and a surface detail of $0.2 \mu\text{m}$. The number of Tau accumulates in the brain were defined using the “spotting” tool for accumulates that had a diameter between $1.5\text{-}2.5 \mu\text{m}$, with a surface detail of $0.36 \mu\text{m}$. The volume of dendritic Tau accumulates was measured using the “surfacing” tool detecting accumulates $\geq 1.0 \mu\text{m}$ defined by surface detail of $0.36 \mu\text{m}$. Thresholding was achieved with local contrast background subtraction with the diameter of the largest sphere which fits into the object. Thresholding was manually adjusted for each animal to capture true structures. Analysis of each cohort was completed under blinded conditions. Statistical analysis was performed using a Two-way ANOVA and a multiple comparisons test.

Quantitative analysis of retinal degeneration was achieved using digital microscope images established ImageJ plugin tool Flynotyper(61) n=6.

SDS-PAGE Western blotting procedure

Tissue preparation: This study utilised the GMR.ninaE-Gal4 driver. Flies were collected at 18°C and moved to 29°C to age for 2 days for total Tau expression and 14 days for measuring p-Tau levels. Flies were then snap frozen in liquid nitrogen and decapitated, in preparation for homogenisation. The heads and brains were then homogenised in an Eppendorf using a pestle for 3 minutes in ice-cold fly homogenising buffer (FHB) containing 10mM Tris, 150mM NaCl, 5mM EDTA, 50mM NaF, 1mM Na₃VOF₃, 5mM NaPPi, 4mM Urea, 5mM DDT, Glycerol, Protease inhibitor (1:100) and Phosphatase inhibitor (1:100). 3ul of FHB was used per head. Lysates were then centrifuged at 10,000 x g for 5 mins at room temperature. Once centrifuged, the supernatant was collected and boiled with 2X SDS-PAGE sample buffer in a 1:1 ratio at 95°C for 5 minutes in a sterile Eppendorf.

Western blots: Proteins were resolved using vertical electrophoresis on 1.5mm 10% SDS-PAGE acrylamide hand-cast gels at 120V for 90 minutes. **For total Tau expression:** 15 male flies were collected for each set of repeats for each sample group. Each well used contained 25µL of the fly homogenate sample buffer mix. Page Ruler Protein Plus ladder (Thermofisher Scientific) was added to each gel. After electrophoresis, the gel containing resolved proteins were electro-transferred onto a nitrocellulose membrane using the electroporator at 60V for 90 minutes, with an icebox to maintain ~4°C. Following electro-transfer, the membranes were immediately placed in blocking buffer containing 5% bovine serum albumin (BSA), 0.1% tween using 1X PBS on a shaker for 60 minutes. Following blocking, the membranes were then incubated with the primary antibodies in a cold room shaker maintaining 4°C overnight. Antibodies listed in table 1 were made up in the blocking buffer at the given concentrations.

Actin was the loading control used in all experiments. The membranes were then washed 3 times with 1X PBS 0.5% tween for 5 minutes on a shaker. Lastly, the membrane was incubated in Anti-Rabbit-800 (Licor, #D00804) and Anti-Mouse-680 (Life technologies A21057) (1:20,000), and washed 3 times with 1X PBS 0.5% tween, for 5 minutes. The membranes were then visualised using LiCOR imager, and analysed using ImageJ software. **Statistical Analysis:** Western blot bands were quantified using ImageJ software. Tau bands were measured at ~100kDa and Actin bands were measured ~40kDa.

Sarkosyl solubility assay to enrich for Tau fibrils: Adapted from Colodner et al., 2010 and Goedert et al., 1992. Briefly, 164 heads (50:50 male:female) reared at 25°C and aged at 29°C for 14 days were collected and snap frozen. Heads were taken and homogenised in 200µl of buffer (15mM NaCl 25mM Tris-HCl at pH 7.4, 1mM EGTA, and 1mM EDTA) containing protease cocktail inhibitor. Samples were then centrifuged for 2 mins at 1000g, and debris pellet was discarded. The supernatant homogenate was then spun for 1 hour at 100,000g at 25°C, and the resulting supernatant was saved as soluble fraction (S1). The pellet was resuspended in a salt/sucrose buffer to wash (10% sucrose, 0.8M NaCl, 10mM Tris-HCl at pH 7.4, and 1mM EGTA) containing protease cocktail inhibitor, and centrifuged for 30 mins at 15,000g at 25°C. Sarkosyl was added to the supernatant (to make 1% Sarkosyl) and incubated at 37°C for 1 hour and then centrifuged for 2 hours at 100,000g. The Sarkosyl soluble fraction was saved (S2), and the pellet was resuspended in 15µl of homogenisation buffer and saved as Sarkosyl insoluble fraction (S3). All samples were diluted in 2 x Laemmli buffer and boiled for 5 mins at 95°C and run on a SDS-polyacrylamide gel. Equivalent volumes of samples were loaded. For each solubility assay the Tau band was quantified in Image Studio Lite.

Microtubule binding assay: Briefly, 10 male heads from flies reared at 25°C and aged at 29°C for 14 days were collected and snap frozen. For each condition, heads were homogenised in 50µl microtubule-binding assay buffer containing protease, kinase and phosphatase inhibitors

(100 mM MES pH 6.8, 500 μ M Mg SO₄, 1 mM EGTA, 4 mM DTT, 2 mM dGTP, 20 μ M taxol, 0.1% triton-X 100, 30 mM NaF, 20 mM sodium pyrophosphate, 40 mM 2-glycerophosphate, 3.5 mM sodium orthovanadate, 10 μ M staurosporine and protease inhibitor cocktail). Samples were then centrifuged for 2 mins at 1000g, and debris pellet was discarded. Homogenates were centrifuged at 12,000 g for 1 h at 4°C; the pellet representing the fraction enriched for microtubule-bound proteins was resuspended in 35 μ l of homogenisation buffer. All samples were diluted in 2 x Laemmli buffer and boiled for 5 mins at 95°C and run on a SDS-polyacrylamide gel.

Climbing assay: Climbing behaviour was performed using 5 cohorts of 10 adult male flies (n= 50) with pan-neuronal expression (*Elav*-GAL4) of hTau2N4R and Oregon R WT as a control (WT). Flies were placed in cylinders in an assay room with controlled lighting conditions, temperature (23 °C) and humidity (30–40%). The assay was conducted by tapping flies 3 times upon a mouse pad to send the flies to the bottom of the cylinder and recording the flies climbing up the sides of the measuring cylinder. The distance climbed by the flies was recorded at 10 seconds. The assay was repeated three times, with 2 minute rest between each trial and the mean was calculated. Crosses were set on RI-AG03 enriched diet, and progeny were aged on the drug throughout the experimental period at either a low (LD) and high (HD) dose of the RI-AG03 tau aggregation inhibitor (0.08 μ M and 0.8 μ M). These concentrations were selected after preliminary studies (40). 2-way ANOVA was used for Statistical Analysis via Graphpad Prism Software.

Table 1: Table of antibodies used in immunolabeling for microscopy, and western blot immunodetection

Further antibody classification as either rabbit/polyclonal (Rb) or mouse/monoclonal (Ms). Phosphoepitopes detected at serine (Ser) or threonine (Thr) residues.

Antibody	p-Tau Epitope detected	Vendor and catalogue number	Concentration	Rb/Ms/Chk target
Anti-human Tau	Total tau	Agilent (Dako) A0024	1:15000 Western blots 1:1000 microscopy	Rb
AT180	pThr231	Thermo Scientific Fisher MN1040	1:250	Ms
AT100	pThr212 pSer214	Autogen Bioclear UK	1:250	Ms
AT8	pSer202 pThr205	Thermo Scientific Fisher MN1020	1:800	Ms
AT270	pThr181	Thermo Scientific Fisher	1:800	Ms
pS262	pSer262	Thermo Scientific Fisher 44-750G	1:500	Rb
PHF-1	pSer396 pSer404	Peter Davies (ALZ Forum)	1:500	Ms
Anti-tau (Tau-22)	Oligomeric tau	Thermo Scientific Fisher	1:500	Rb
mActin	N/A	Sigma- Aldrich	1:3000	Ms
rActin	N/A	Cell signalling Technology	1:3000	Rb
Anti-rabbit 800	N/A	LiCor	1:20 000	Rb
Anti-mouse680	N/A	LifeTechnology	1:20 000	Ms
anti-chicken Alexa 488	N/A	Thermo Scientific Fisher	1:500	Chk
Anti-rabbit Alexa 555	N/A	Thermo Scientific Fisher	1:500	Rb
α -GFP	N/A	Thermo Scientific Fisher	1:1000	Chk

Table 2: Table of *Drosophila* stocks, genotypes, and source.

<i>Drosophila</i> strain	Full genotype	Source	ID	Description/use
Or47b-GAL4	; Or47b-GAL4,UAS-mCD8::GFP/Cyo;	Professor Liquan Luo (Stanford University, CA, USA).	N/A	For expression of Tau variants in a small bundle of olfactory sensory neurons (Odorant receptor 47b – VA1d glomeruli).
GAL4-ninaE.GMR	;GAL4-ninaE.GMR/Cyo;;	Balanced from Bloomington stock	FBti0002994 BL1104	Retinal driver +/+; GAL4-ninaE.GMR/Cyo; +/+ for strong expression of Tau variants in photoreceptor cells, used in retinal imaging (SEM and digital microscopy) and used in the western blot immunoassays.
Elav-Gal4 C155	P[w [+mW.hs] =GawB]elav[C155]	Bloomington	BL458	Pan-neuronal driver used in behavioural study of locomotion; climbing assay
Tau2N4R	+/+; UAS-mCherry::hTau2N4R/Cyo;+/+	Allan Lab, UBC	N/A	10x UAS mCherryTag on N-terminal of Tau Insertion into Attp40 site
Tau2N4R ^{Δ306} VQIVYK311	+/+;UAS-mCherry::hTau2N4R ^{Δ30} 6VQIVYK311/Cyo;+/+	Allan Lab, UBC	N/A	10x UAS mCherryTag on N-terminal of Tau Insertion into Attp40 site

Tau2N4R ^{E14}	+/+;UAS- mCherry::hTau2N4R ^{E14} /Cyo;+/+	Allan Lab, UBC	N/A	10x UAS mCherryTag on N-terminal of Tau Insertion into Attp40 site
Tau2N4R ^{E14} - Δ306VQIVYK311	+/+;UAS- mCherry::hTau2N4R ^{E14} -Δ306VQIVYK311/Cyo;+/+	Allan Lab, UBC	N/A	10x UAS mCherryTag on N-terminal of Tau Insertion into Attp40 site

Conflict of interest

The authors declare they have no conflict of interest.

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Declaration

During the preparation of this work the author(s) used Claude AI/Anthropic as an editorial tool in order to improve readability and reduce word count. After using this tool/ service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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Figures

Figure 1

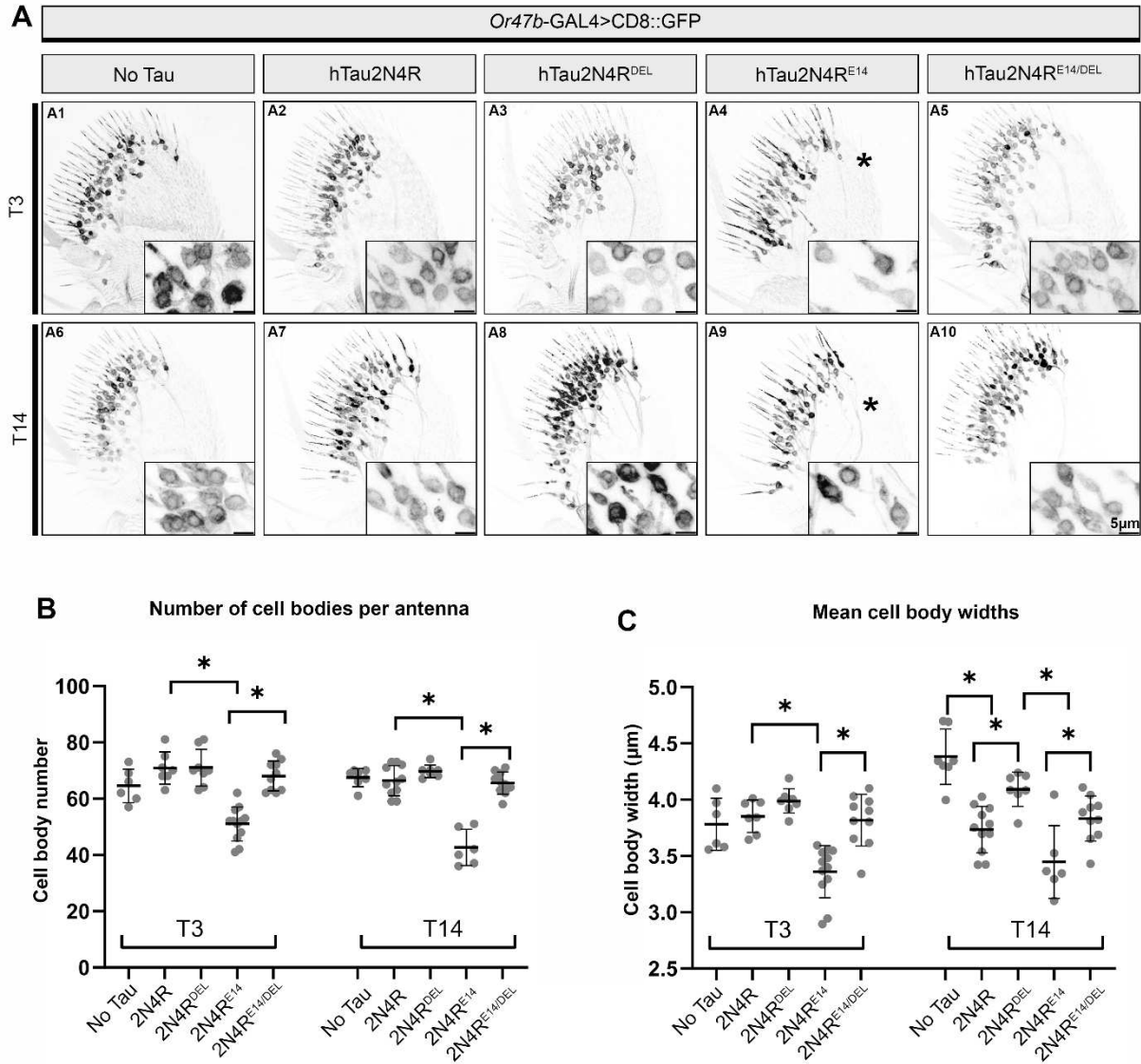
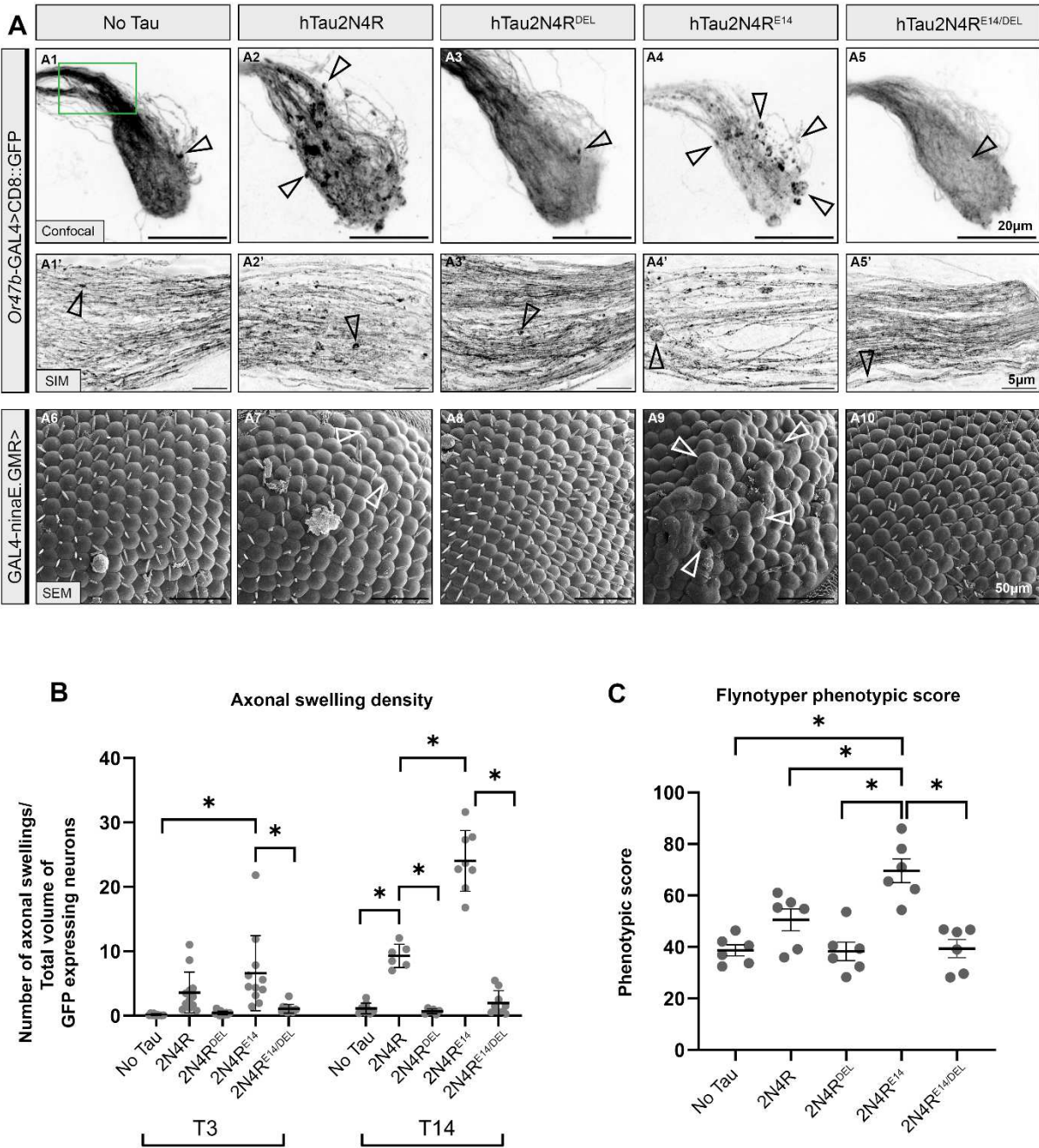


Figure 1. Phosphorylation dependent somato-dendritic degeneration requires aggregation.

(A) Maximum projection confocal images of the adult antenna of flies expressing membrane bound GFP (CD8::GFP) at the Or47b neurons. Columns represent genotypes expressing different mCherry-tagged hTau2N4R variants plus the driver alone expressing GFP only as a control (left most column). Rows represent post-developmental age (3 or 14 days). Magnified projections (30 slice depth) of the soma region are included. Asterisk shows the reduced soma density induced by hTau2N4R^{E14} expression (A9), which is rescued in hTau2N4R^{E14/DEL} (A10).

(B-C) Quantification of cell bodies per antenna (B) and mean cell body width (C) for the indicated genotypes and time points. Cell bodies were manually counted and measured using ImageJ. Graphs represent mean $\pm$ SD, and dots represent averages per antenna. * = $p < 0.05$ (2-way ANOVA with Tukey's multiple comparisons). N = 6-12 antennae collected from two biological repeats per genotype.



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1085 **Figure 2. Phosphorylation dependent axonal and synaptic degeneration requires aggregation.**

1086 (A) Maximum projection confocal (A1-5) or SIM (A1'-5') images of the brain and antennal lobe of
1087 flies expressing membrane bound GFP (CD8::GFP) at the Or47b neurons enhanced with α -GFP.
1088 Columns represent genotypes expressing different mCherry-tagged hTau2N4R variants plus the driver
1089 expressing GFP only as a control (left most column). Rows represent post-developmental age 14 days.
1090 Normal structure of the neurons shows some axonal swellings (Arrows) that occur with age and are
1091 increased in WT hTau2N4R flies (A2) and exacerbated in hTau2N4R^{E14} expressing flies (A4). Green
1092 inset box on A1 highlights the Or47b glomeruli commissure, imaged with SIM (A1'-5') in the middle
1093 row.

Bottom row of images show SEM micrographs (**A6-A10**) of day 14 *Drosophila* eyes expressing hTau2N4R mutants using the GAL4-ninaE.GMR driver (at 1600x magnification). Abnormal retina and degenerated cells are labelled with arrowheads.

(B) Quantification of axonal swellings were automatically quantified using 3D images with IMARIS (see methods). Graphs represent mean $\pm$ SD, and dots represent individual values. * = $p < 0.05$ (2-way ANOVA with Tukey's multiple comparisons). N= 6-14 brains collected from two biological repeats per genotype.

(C) Quantification of retinal degeneration using a phenotypic scoring system Flynotyper plugin. Graphs represent mean $\pm$ SD, and dots represent individual values. * = $p < 0.05$ (2-way ANOVA with Tukey's multiple comparisons). N=6 per genotype.

Figure 3

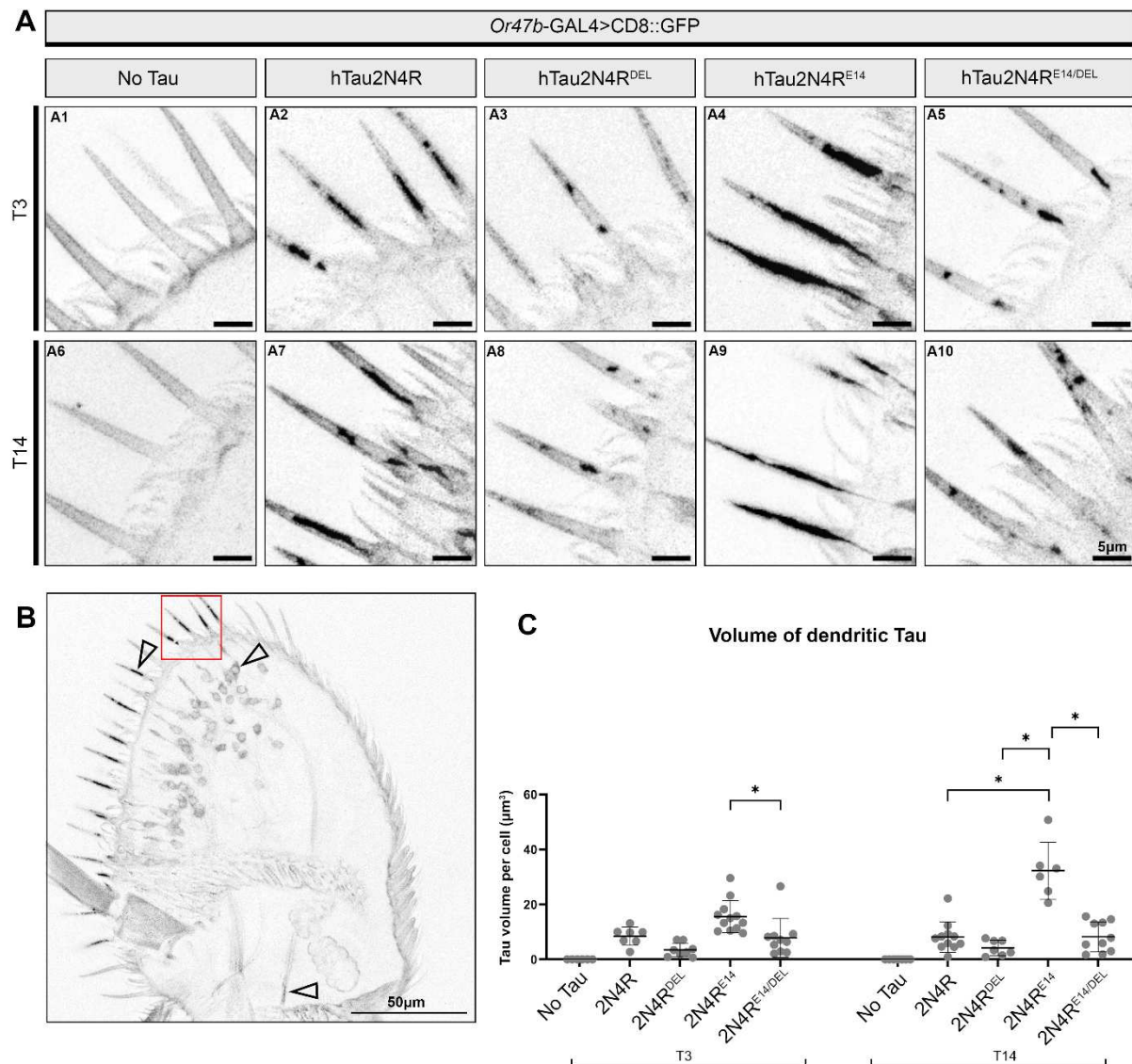


Figure 3. Phosphorylation dependent dendritic Tau accumulation requires aggregation.

(A) Midline 30 slice stacked confocal images of the adult antenna of flies labelled by the mCherryTau signal from expressed mutants in the *Or47b* neurons. Columns represent genotypes expressing different mCherry-tagged hTau2N4R variants plus the driver expressing GFP only as a control (left most column). Rows represent post-developmental age (3 or 14 days). Magnified regions of interest over the dendrites are presented (A1-A10).

(B) Example image of whole mount antenna, of A2. Arrows point towards Tau expression present within the dendrites, somas and axons. Other whole mounts can be viewed in supplementary figure 6.

(C) Quantification of the volume of dendritic Tau in the antenna was quantified using 3D projection images with IMARIS with a surfacing tool using N=6-12 from two biological repeats. Graphs represent Mean $\pm$ SD. * = $p < 0.05$ (2-way ANOVA with Tukey's multiple comparisons).

Figure 4

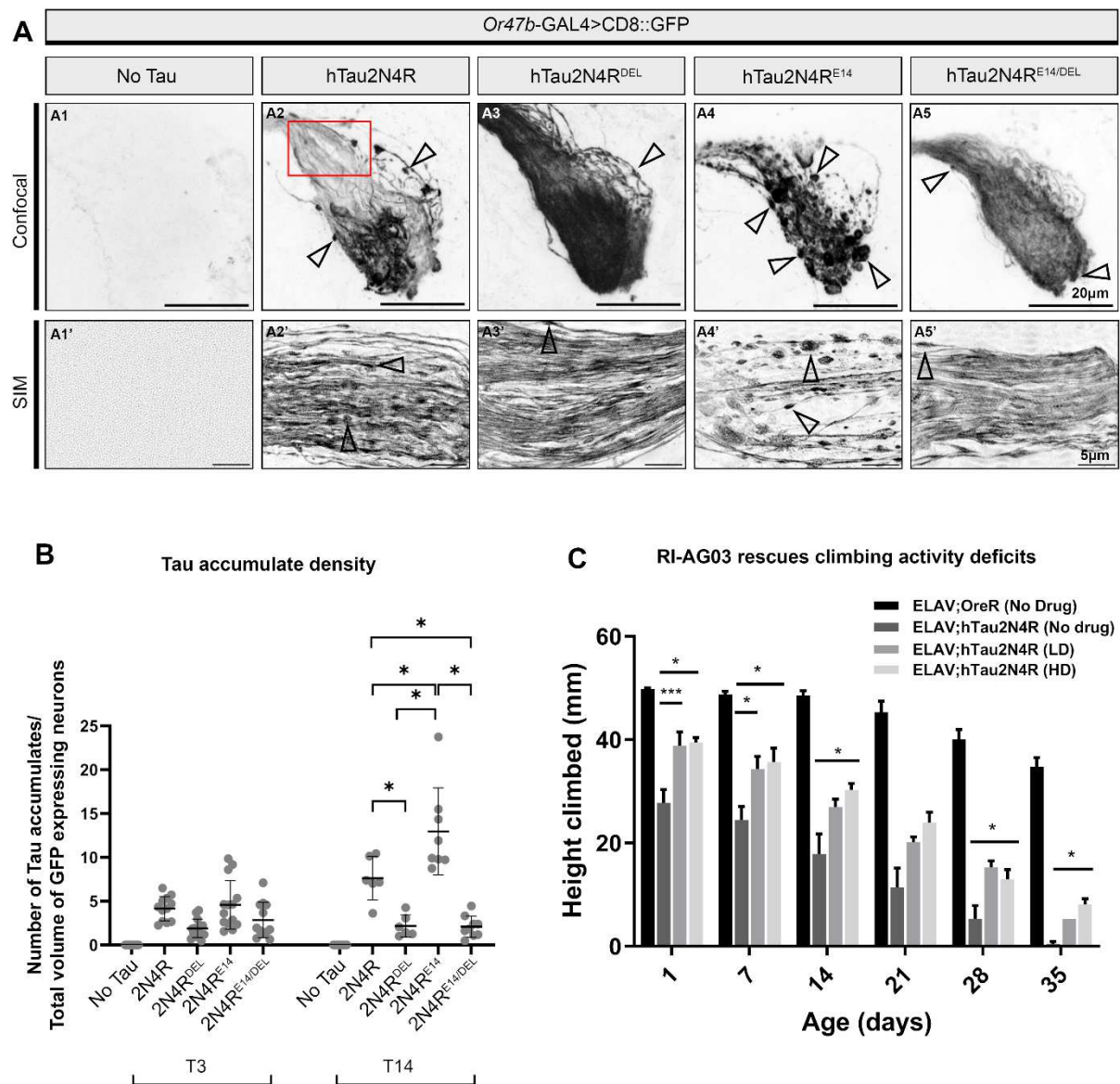


Figure 4. Phosphorylation dependent axonal and synaptic Tau accumulation requires aggregation, and treatment of aggregation inhibitor RI-AG03 rescues locomotive dysfunction in Tau-expressing *Drosophila*.

(A) Maximum projection confocal (A1-5) or SIM (A1'-5') images of the antennal lobe in the brain of day 14 old flies expressing mCherry::Tau mutants in the *Or47b* neurons (enhanced with α -hTau Dako). Arrows point toward beaded Tau accumulates within expressing neurons which increase in frequency and size with age and are exacerbated in hTau2N4R^{E14} expressing flies (A4), but are significantly suppressed in hTau2N4R^{DEL} (A3) and hTau2N4R^{E14/DEL} (A5). Red inset box on A2 highlights the *Or47b* glomeruli commissure, imaged with SIM (A1'-A5') at T14 on the bottom row.

(B) Quantification of the Tau accumulate density were quantified using 3D images with IMARIS with a spotting tool (see methods) N=6-14 from two biological repeats. Graphs represent mean $\pm$ SD. * = $p < 0.05$ (2-way ANOVA with Tukey's multiple comparisons).

(C) Climbing assay was used to assess locomotive function of pan-neuronal hTau2N4R expressing flies treated with RI-AG03. Bar graph showing the average height climbed by each group (5 cohorts of 10

1159 male flies) over a 35 day period. Control group (ELAV;OreR) and hTau2N4R-expressing flies were
1160 subjected to low-dose (0.08uM) (LD) and high-dose (0.8uM) (HD) treatments of the aggregation
1161 inhibitor RI-AG03 from 1 to 35 days of age. hTau2N4R-expressing flies exhibited a significant
1162 reduction in normal climbing activity compared to control flies. Treatment with RI-AG03 at both LD
1163 and HD significantly improved the climbing performance of hTau2N4R-expressing flies. Error bars
1164 represent mean $\pm$ SEM. * = $p < 0.0001$ (2-way ANOVA with Tukey's multiple comparisons).

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Supplementary Files

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- [Cooper2026supplementaryfigures.pdf](#)