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Cell type-specific processing of human Tau proteins in Drosophila

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Abstract Accumulation of hyperphosphorylated Tau is associated with a number of neurodegenerative diseases collectively known as tauopathies. Differences in clinical and cognitive profiles among them suggest differential sensitivity of neuronal populations to Tau levels, phosphorylation and mutations. We used tissue specific expression of wild type and mutant human *tau* transgenes to demonstrate differential phosphorylation and stability in a cell type-specific manner, which includes different neuronal types and does not correlate with the level of accumulated protein. Rather, they likely reflect the spatial distribution or regulation of Tau-targeting kinases and phosphatases.

Keywords: Tau; Phosphorylation; Tissue specificity; Drosophila

1. Introduction

Tau is a microtubule binding protein, preferentially distributed in the axons of the central (CNS) and some neurons of the peripheral (PNS) nervous system [1]. In humans, six Tau isoforms arise via tissue and temporally regulated alternative splicing that yield proteins with 3 or 4 microtubule binding repeats (3R, 4R) and different amino-terminal sequences (0–2N) [2]. Tau is extensively phosphorylated either constitutively [3,4], or facilitated (primed) at particular sites, by prior phosphorylation [5]. Tauopathies are a diverse group of neurodegenerative dementias characterized by intraneuronal aggregation of hyperphosphorylated Tau [3,4,6]. Mutations in the single *tau* gene cause hereditary Frontotemporal Dementia with Parkinsonism linked to chromosome 17 (FTDP-17) [4]. In contrast, accumulation of hyperphosphorylated normal Tau yields intracellular aggregates varying in regional distribution and morphology (Neurofibrilar Tangles (NFTs) and Paired Helical Filaments (PHFs)) that characterize Alzheimer's (AD), Pick's Disease and other tauopathies [2,3]. Tauopathies are differentiated by their unique clinical and cognitive profiles, likely reflective of the regional distribution of CNS pathology [2,7]. This is indicative of the differential sensitivity of particular neuronal groups to Tau levels, phosphorylation and mutations [6]. Cell type-specific aggregate formation is a probable consequence of differential distribution of kinases and phosphatases that target Tau [5,8,9]. Although, the human data indicate that all CNS and PNS neurons are not equally susceptible to pathogenesis and this differential sensitivity is important for diagnosis [2,7,10], it tends to be overlooked in tauopathy models.

The genetic versatility of Drosophila has advanced our understanding of Tau toxicity, age-dependent neurodegeneration [11–14] and learning and memory and axonal transport defects [15,16]. In flies, NFT formation required Tau co-expression with Tau-targeting kinases such as GSK-3β [12,17,18]. Even then, the degree of aggregate formation and their characteristics appeared to vary in a cell type-dependent manner [11,12,17]. Here, we address the hypothesis that Tau proteins are differentially processed in particular Drosophila tissues and neuronal populations. Because hyperphosphorylation is the main characteristic of pathological Tau, we focused on phosphorylation patterns in the tissues targeted in most Drosophila models, the retina and the nervous system.

2. Materials and methods

2.1. Drosophila culture and strains

Drosophila were cultured in soy flour and $CaCl_2$-supplemented sugar–wheat flour food. Gal4 driver lines were: the pan-neural driver ELAV, the retina-specific GMR, the mushroom body driver c772 [15] and the larval motor neuron driver D42-Gal4 [19]. UAS-*htauWT*, UAS-*htauR406W*, UAS-*htauV337M* transgenics [11] were provided by M. Feany, while the UAS-*htauR406WS2A* [17] was obtained from B. Lu and UAS-*btau* from Ito [20].

2.2. Western blotting and antibodies

Tissues from 2 to 3-day-old adult females and 3rd instar larvae were homogenized in 1× Laemmli buffer (50 mM Tris pH 6.8, 100 mM DTT, 5% 2-mercaptoethanol, 2% SDS, 10% glycerol and 0.01% bromophenol blue). The lysates were heated for 10 min at 95 °C, centrifuged at $8000 \times g$ for 5 min and separated in SDS–acrylamide gels. Because of varying affinities of anti-Tau antibodies and efficiency of Gal4 drivers, the amount of extract loaded per lane for best resolution was determined experimentally for each antibody. Proteins were transferred to PVDF membranes and probed with the monoclonal antibodies: anti-Tau (TOTAL) (Zymed laboratories) at 1:1000, AT100 (Pierce Endogen) at 1:250 and the polyclonal antibodies pS262 (Biosource) at 1:500 and pS396 (Biosource) at 1:2000. Monoclonal antibodies AT8 (used at 1:200), AT180 (used at 1:200) and TAU 1 (used at 1:2000) were kindly provided by A. Mudher. To normalize for sample loading, the membranes were concurrently probed with an anti-syntaxin monoclonal antibody (8C3, Developmental Studies Hybridoma Bank) at a 1/2000. Bovine Tau was detected with a monoclonal antibody (Sigma), not cross-reacting with human Tau at 1:1000. A rabbit polyclonal anti-Beta-Galactosidase antibody (Rockland) was used at 1:1000. Proteins were visualized with chemiluminescence.

2.3. In vitro dephosphorylation

Heads were homogenized in RIPA (137 mM NaCl, 20 mM Tris pH 8.0, 10% glycerol, 0.1% SDS and 0.1% sodium deoxycholate) supplemented with protease inhibitors (Sigma). Twenty micrograms of total protein was treated with 400 U λ-phosphatase (New England Biolabs)

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at 30 °C for 30 min according to manufacturer's instructions. One-third of each sample was resolved by SDS–PAGE and subjected to western blot analysis.

3. Results

3.1. Differential accumulation of human tau transgenic proteins in the Drosophila eye and brain

We used *htauWT* transgenics because the wild type (WT) Tau accumulates in heritable and sporadic AD and the FTDP-17-linked mutations *R406W* and *V337M* [11], because FTDP-17 has distinct distribution and symptoms from AD [4,21]. Finally, the triple mutant *R406WS2A* bears mutations in sites reported essential for primed Tau phosphorylation in R7 photoreceptors [17] and was used to probe utilization of these sites in other cell types. To facilitate the analysis and inter-transgene comparisons, all transgenic proteins were of the 1N4R isoform. We compared tissue specific Tau accumulation using the UAS/Gal4 system [22] and the GMR and ELAV drivers because they are commonly used in Drosophila Tauopathy models [13,14,23] and both direct Tau accumulation in tissues, where the Drosophila ortholog is found [24]. GMR is expressed in neurons of the retina including photoreceptors, but also in non-neuronal structural and supporting cells [25]. ELAV directs expression in all CNS and PNS neurons [26].

WT and mutant Tau in the retina appeared as a higher than the predicted size multi-species broad band (Fig. 1A). Similar amounts of WT and V337M accumulated, while R406WS2A appeared elevated and R406W relatively reduced. In contrast, Tau accumulating in neurons appeared as a single species slightly larger than the biggest species under GMR (Fig. 1A). Considering the large number of neurons accumulating Tau in heads, the protein levels suggest that ELAV is a weaker driver than GMR. Strikingly, the R406WS2A protein which exhibited the highest accumulation under GMR was nearly absent under ELAV, indicating that the S262 and S356 to A mutations [17] render it less stable in neurons. The multi-band protein accumulation under GMR was also exhibited by a bovine Tau, which appeared as a single band in neurons. In contrast, like green fluorescent protein (GFP) and other exogenous proteins (not shown), β-galactosidase accumulated as a single species under both drivers (Fig. 1B). These data indicate that the distinct protein profiles do not reflect inherent differences in processing or stability of all non-Drosophila proteins in the two tissues, but rather are specific to Tau.

The multiple species in the retina could reflect partially phosphorylated or otherwise modified proteins. Alternatively, they could be degradation or incomplete translation products. To resolve this, Tau proteins accumulating in the retina or neurons were subjected to *in vitro* de-phosphorylation with λ-phosphatase. Tau in the nervous system and the largest species in the retina exhibited an apparent molecular size of 60–62 kD. De-phosphorylation yielded a single band of 48–50 kD, irrespective of whether WT or mutant Tau accumulated in the retina or neurons (Fig. 1C). Smaller than full length de-phosphorylated proteins were not observed, even upon over-exposure of the blots or probing with additional anti-Tau antibodies (not shown). These results are consistent with the notion that the multiple Tau species in the retina were not products of degradation or abortive translation. Hence, while

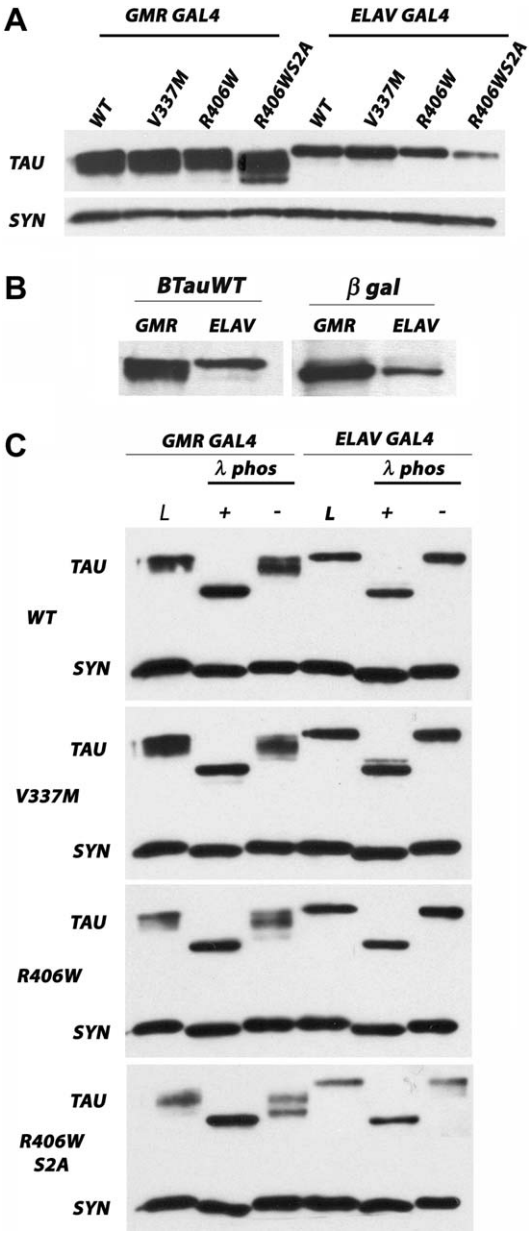

Fig. 1. Distinct patterns of Tau accumulation in the retina and neurons reflect phosphorylation differences. (A) A representative semi-quantitative Western blot of head lysates from flies accumulating WT and mutant Tau in the retina (GMR-GAL4) and nervous system (ELAV-GAL4), probed with an antibody that recognizes all human Tau species (TOTAL antibody). Three heads were loaded per lane. Drosophila anti-syntaxin antibody (SYN) was used to monitor loading. (B) Bovine Tau (BTauWT) and, β-galactosidase (βgal) were similarly expressed in the retina and neurons with GMR and ELAV. Equal amounts of protein were loaded per lane. (C) Equal amount (6.5 μg) of head lysates from animals accumulating WT and mutant Tau proteins under ELAV and GMR-Gal4 were subjected to *in vitro* dephoshorylation by λ-phosphatase (λ-phos, +). The reactions were analyzed by Western blotting using the TOTAL Tau antibody (TAU) alongside with equal amount of head lysates without phosphatase (−), or untreated lysates (L). Equivalent amounts of protein were used per lane as probing with SYN verified, while phosphate removal from SYN in treated samples (+) yielding smaller species ascertained phosphatase activity.

Tau is maximally phosphorylated in neurons, phosphorylation in the retina appeared incomplete, yielding multiple bands. Note that although equivalent amounts were used, incubation

without λ-phosphatase appeared to reduce Tau in the sample. This was apparent for proteins accumulating in the retina and to a lesser degree with Tau in neurons. This suggested that partially phosphorylated Tau may be more vulnerable to endogenous proteases than maximally phosphorylated, or dephosphorylated protein. In the nervous system, this may account for the reduced stability of the R406WS2A protein.

3.2. Distinct phosphorylation of Tau in the retina and the nervous system

We used phospho-specific antibodies to uncover differentially phosphorylated epitopes in the retina and nervous system (Fig. 2A). Because phosphorylation at S202/T205, is frequently associated with AD, its status was probed with antibody AT8 [3] and was found occupied both in the retina and the nervous system. This site is targeted by cdk-5 and GSK-3β, but was reported to require priming phosphorylation at S262 and S356 by the kinase Par1 in R7 photoreceptor neurons [17]. Significantly, although reduced in comparison to WT, the R406WS2A protein lacking these Par1 sites appeared phosphorylated in the retina. Furthermore, despite the much reduced R406WS2A accumulation in neurons, its phosphorylation on S202/T205 was clearly not eliminated indicating that it occurred in non-R7 cells of the retina and CNS neurons.

These results were confirmed using the TAU1 antibody, which recognizes unphosphorylated S202/T205. As expected, the majority of R406WS2A protein in the retina appeared unphosphorylated at that site. In contrast, low signal was detected on R406WS2A in the nervous system, confirming that despite its low abundance most of the protein is phosphorylated on S202/T205. In addition, multiple species were identified by this antibody when Tau accumulated in the retina, indicating that S202/T205 remained unphosphorylated in a large fraction of WT and mutant proteins. In neurons, the antibody recognized an apparent single species lacking S202/T205 phosphorylation on WT and mutant proteins. This indicates that the band detected with the TOTAL antibody contains at least two species differing in S202/T205 phosphorylation, a size difference too small to resolve in our gels.

A major phosphorylation difference was uncovered at T212/S214, which is recognized by the AT100 antibody and targeted by GSK-3β [3]. Minimal phosphorylation was detected on Tau accumulating in the retina and was absent from R406WS2A (verified by longer exposure of the blots – not shown). The low level of T212/S214 phosphorylation on WT and mutant proteins in the retina likely occurred in photoreceptor neurons because immunostaining with AT100 has been reported to reveal a diffuse signal for WT Tau in these cells [13]. In contrast, T212/S214 appeared phosphorylated on Tau accumulating in neurons, except for R406WS2A. This is highly significant, because T212/S214 phosphorylation constitutes a tauopathy-specific epitope and is linked to the presence of NFTs, or the potential to form them [8,12]. The prominent T212/S214 phosphorylation on Tau in neurons suggested that GSK-3β is either distributed preferentially, or the phosphorylation is more stable in neurons compared to cells of the retina.

Phosphorylation at T231/S235 by GSK-3β [27] has been reported to affect microtubule binding [2] and this epitope, often associated with tauopathies is recognized by the AT180 antibody [3]. T231/S235 was strongly phosphorylated on Tau accumulating in the retina and the nervous system. In contrast,

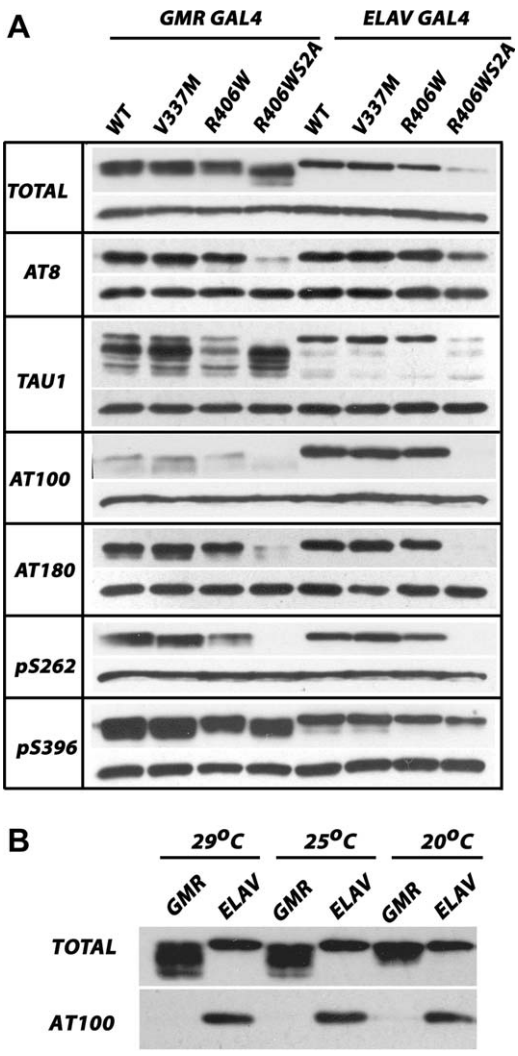

Fig. 2. Survey of key phosphorylation sites on Tau proteins in the retina and nervous system. (A) Western blots from head lysates of animals accumulating the indicated WT and mutant proteins in the retina and brain were probed with the TOTAL Tau antibody and phospho-specific antibodies to investigate phosphorylation at sites progressively from amino to carboxy-terminus: AT8, AT100, AT180, pS262 and pS396. TAU1 was used to detect non-phosphorylated Tau at the positions recognized by the AT8 phospho-antibody. SYN was used to ascertain equivalent loading (lower band in all panels). To normalize for the different affinities of the anti-Tau antibodies, the number of female heads used per lane was adjusted as follows: anti-total Tau, 3 heads/lane; AT-8, 1head/lane; AT100, 7 heads/lane; AT180, 1 head/lane; pS262, 7 heads/lane; pS396, 1 head/lane; TAU1, 1 head/lane. (B) *htauWT* expressing flies under the GMR or ELAV drivers were raised at the indicated temperatures to manipulate the amount of transgenic protein produced. The total amount of Tau (TOTAL) and that phosphorylated at positions 212/214 (AT100) was assessed in the corresponding head lysates (3 heads/lane for anti-TOTAL Tau and 7 heads/lane for AT100).

phosphorylation at this site on R406WS2A was barely detectable in the retina and absent in the nervous system. These results are in agreement with the proposed requirement for priming by Par1 for subsequent GSK-3β-mediated phosphorylation at T231/S235 [17]. Indeed, phosphorylation at the S262 Par-1 site [17], was eliminated on R406WS2A, but not other Tau proteins both in the retina and the nervous system as expected. An additional unprimed GSK-3β or cdk5 phos-

phorylation site outside the microtubule binding domain, is S396 [27] and is associated with tauopathies and PHF formation [2,6]. S396 was phosphorylated on all Tau proteins accumulating in the retina and neurons. However, given the level of R406WS2A in the retina, S396 phosphorylation appeared mildly reduced compared to that on WT. In contrast, even though much reduced in neurons, all R406WS2A appeared phosphorylated at this site.

Collectively, the results indicate differential phosphorylation on a number of sites depending on whether Tau accumulates in the retina or the nervous system and a large fraction of Tau remains partially phosphorylated in the eye. These differences could arise because of the increased level of Tau accumulation under GMR, or cell type-specific differences in the distribution of kinases and phosphatases that target it. Manipulating transgene expression by raising animals at different temperatures [15,28] did not alter the pattern of Tau accumulation, or T212/S214 phosphorylation (Fig. 2B). Therefore, phosphorylation differences do not result from overwhelming responsible kinases with excess transgenic proteins. Rather, the results are consistent with limited distribution of Tau-targeting kinases, or differential regulation of their activities in the tissues and cell types under investigation.

3.3. Neuronal type specific post-translational processing of Tau

To explore the possibility that human Tau is processed differently in various populations of Drosophila neurons, we expressed WT Tau in the mushroom bodies (MBs) using the c772 driver [15,29]. The MBs, are comprised of approximately 5000 CNS neurons essential for olfactory learning and memory [30]. Tau in the MBs accumulated in a profile identical to that under ELAV and was highly phosphorylated at S202/T205 (AT8) and T212/S214 (AT100) (Fig. 3). Longer exposure of the blots did not reveal additional bands, and the pattern did not change if flies were raised at 29 °C (not shown). In contrast, a smaller Tau species accumulated in larval motor neurons [16], which lacked phosphorylation at S202/T205 and T212/S214 (Fig. 3). However, this species contained other modifications since it appeared larger than the 42 kD predicted size of Tau. As expected, the smaller protein was also present in larvae expressing Tau pan-neuronally (ELAV). A larger species, absent from larvae expressing Tau in motor neurons, thus accumulating in all other neuronal types, appeared similar in size to that found in adult CNS. In agreement, S202/T205 and T212/S214 were occupied only on the larger species accumulating in non-motor larval neuronal populations (Fig. 3). Therefore, Tau accumulating in these two different types of larval neurons appears to be processed differently, likely a reflection of the kinases and phosphatases targeting this protein present in each cell type. In agreement with this interpretation, co-expression of GSK-3β in larval motor neurons has been shown to increase Tau phosphorylation [16].

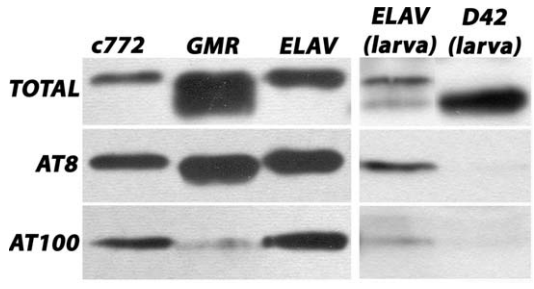

Fig. 3. Specific processing of Tau in different neuronal populations. Differences in phosphorylation pattern of WT Tau accumulating in adult mushroom bodies (c772, 7 heads/lane), in the eye (GMR, 2 heads per lane), the entire nervous system in adults (ELAV, 2 heads/lane) and larvae (1 larva/lane) and larval motor neurons (D42, 1 larva/lane).

These results demonstrate that cell type-specific phosphorylation on particular sites and possibly other post-translational modifications on human Tau occur differentially in different Drosophila neuronal types, potentially mirroring the complexity of Tau post-translational processing in vertebrates [1,2].

4. Discussion

Tauopathy models must differentiate the effects of WT and mutant Tau proteins and take into consideration their cell type-specificity. Comparison of phosphorylations on WT and mutant Tau proteins did not reveal significant interallelic differences for proteins accumulating in the retina, or the nervous system, except for the R406WS2A mutant [17]. Importantly however, we observed significant tissue specific differences in Tau phosphorylation, attributable at least in part to distinct utilization of phosphorylation sites in the retina and neurons. The two most prominent differences uncovered were underutilization of S202/T205 and T212/S214 in the retina, and total lack of these modifications in larval motor neurons in comparison to that in the CNS. Both sites were occupied in MB neurons, where accumulation of the WT protein was linked to learning and memory deficits [15]. Because S202/T205 and T212/S214 phosphorylation was apparently also absent from protein accumulating specifically in R7 photoreceptor neurons [17], collectively these results suggest that this likely characterizes Tau accumulating in the PNS. Therefore, Drosophila CNS processes Tau similar to vertebrate neurons susceptible to AD [2,4] and is processed differently in the PNS, in agreement with human data [2]. Because in the retina and larval motor neurons S202/T205 and T212/S214 sites were unoccupied, these tissues are not optimal AD models since the disease is characterized by phosphorylation of these sites, but rather tauopathies not requiring these specific modifications. In fact, larval motor neurons served to model Tau-dependent impairments in anterograde transport [16,31], a condition common to multiple tauopathies possibly preceding aggregate formation. Both S202/T205 and T212/S214 sites are targeted by GSK3β, a kinase found to accumulate strongly in the adult CNS and retinal cone and pigment cells, but not appreciably in photoreceptor neurons (Supplementary Fig. 1). Therefore, reduced S202/T205 and T212/S214 phosphorylation in cone and pigment cells expressing Tau under GMR, is a likely consequence of limited GSK3β activity, access to these sites, or increased activity of a specific phosphatase.

Surprisingly, the priming phosphorylation at S262 and S356 by Par1 in R7 photoreceptors [17] was not essential for S202/T205 occupation in other neurons. These results indicate that Par1 phosphorylation is not a requisite priming modification in all neuronal types, or it has an allele specific effect in different tissues. In support of the later notion, although over-expressing Par1 in R7 cells enhanced R406W-mediated neurodegeneration [17], it appeared to suppress V337M-mediated degeneration in the retina [18]. Another surprising

difference is the apparent instability of R406WS2A in neurons in contrast to its abundant accumulation in the retina. This suggests tissue or cell type-specific differences in distribution, abundance or activity of proteases that target Tau. Such proteases may be particularly sensitive to Tau unphosphorylated at sites such as S262, perhaps in combination with S202/T205 occupation leading to efficient degradation of R406WS2A in neurons.

Since Tau is processed cell-specifically, ectopic expression of kinases and phosphatases that target it [1,2,9] may enhance or suppress phenotypes of co-expressed Tau non-specifically and yield similar degenerative phenotypes. In fact, co-expression of GSK3β with R406W in R7 photoreceptors, where we cannot detect the kinase, enhanced degeneration and resulted in the formation of NFT-like inclusions [12]. Therefore, the consequences of WT and mutant Tau accumulation and their interacting proteins must be carefully considered in light of the tissue specific effects described herein. On the other hand, the cell type-specific processing of Tau may be capitalized upon to model the distinct cell type sensitivities, pathologies and cognitive effects of different tauopathies in Drosophila.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.07.045.

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