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Cell type-specific processing of human Tau proteins in Drosophila

Sofia Grammenoudi, Stylianos Kosmidis, Efthimios M.C. Skoulakis*

Institute of Molecular Biology and Genetics, Biomedical Sciences Research Centre ''Alexander Fleming'', 34 Fleming Street, Vari 16672, Greece

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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)

*Corresponding author. Fax: +30 210 965 6563.
E-mail address: skoulakis@fleming.gr (E.M.C. Skoulakis).

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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

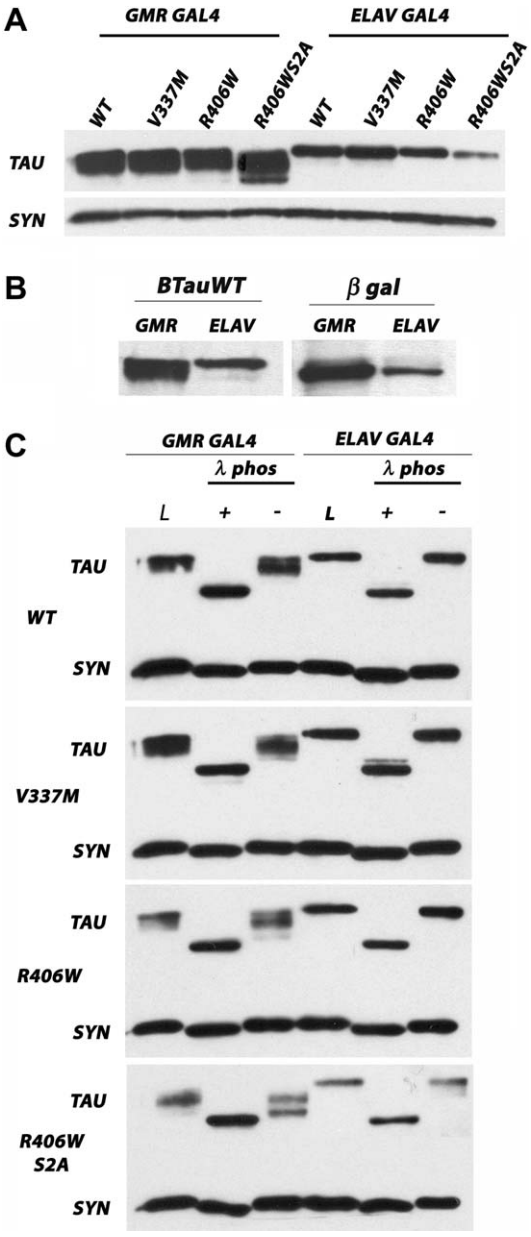

Fig. 1. Distinct patterns of Tau accumulation in the retina and neurons reflect phosphorylation differences. (A) A representative semi-quantitative Western blot of head lysates from flies accumulating WT and mutant Tau in the retina (GMR-GAL4) and nervous system (ELAV-GAL4), probed with an antibody that 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