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Biochemical investigation of Tau protein phosphorylation status and its solubility properties in *Drosophila* ☆

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Abstract

Tau hyperphosphorylation and insoluble aggregate formation are two cellular features of tauopathies. However, the contribution of Tau protein hyperphosphorylation and its aggregation to Tau pathology still remain controversial. Overexpression of human *tau* transgenes in the *Drosophila* eye is toxic and causes neuronal degeneration. We showed that human Tau protein was phosphorylated by endogenous protein kinases in flies, and overexpression of either GSK3β or Cdk5 enhanced *tau*-induced toxicity. Using a dominant-negative approach, we showed that kinase activity is important for the enhancement of *tau*-induced toxicity. Interestingly, such enhancement was accompanied with hyperphosphorylation and alteration of protein solubility properties of Tau. This situation was reminiscent of that observed in pre-tangle neurons in tauopathies patients. We also observed age-dependent Tau aggregate formation in aged transgenic flies. In summary, *tau*-induced toxicity is enhanced when the human Tau protein undergoes hyperphosphorylation, and we further demonstrated that aging contributes to Tau aggregate formation. Our data also underscore the utilization of transgenic *Drosophila* Tau models for the studies of pre-tangle events in tauopathies.

Keywords: Alzheimer's disease; Cdk5; GSK3β; PHF; Pre-tangles

Tau is a neuron-specific microtubule-associated protein localized in the axon of neurons. It is responsible for the assembly of tubulin dimers into microtubules [1]. In healthy neurons, Tau is soluble, non-filamentous, and associated with microtubules [2]. Tauopathies, including Alzheimer's disease (AD), are a group of neurodegenerative diseases characterized by extensive neurofibrillary pathology [3]. Under pathologic conditions, Tau protein is hyperphosphorylated, and its affinity to microtubules reduces [4]. Hyperphosphorylated Tau protein released from microtubules accumulates diffusely in the cytoplasm of neurons, and they are characterized as pre-tangles [5]. These pre-tangles subsequently form paired helical filaments (PHFs), which are components of neurofibrillary tangles (NFTs) found in tauopathies patients [5]. It is also found that there is a close correlation between pre-tangles and *tau*-induced toxicity [5]. Apart from Tau protein hyperphosphorylation, specific mutations in the *tau* gene (including R406W, V337M, and P301L) are also pathogenic [6,7].

A number of protein kinases have been shown to phosphorylate the Tau protein, and they are referred to as Tau protein kinases. They include glycogen synthase kinase 3β (GSK3β), cyclin-dependent kinase 5 (Cdk5), and the mitogen-activated protein kinase family [8]. The longest form of the human Tau protein contains ~80 serine or threonine residues [9], and at least 25 of them are confirmed to be phosphorylated in PHF-Tau [10]. In line with this, protein kinases have also been detected to physically associate with pathogenic Tau in tauopathies patients [11–14]. Other

☆ *Abbreviations:* AD, Alzheimer's disease; Cdk5, cyclin-dependent kinase 5; FTDP-17, frontotemporal dementia with Parkinsonism linked to chromosome 17; GSK3β, glycogen synthase kinase 3β; NFT, neurofibrillary tangles; PHF, paired helical filaments.

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reports also demonstrate that additional Tau protein posttranslational modifications, such as glycation [15], oxidation [16], ubiquitination [17], and sulfo-glycosaminoglycan modification [18], also contribute to PHF/NFT formation.

Transgenic models for tauopathies have been established, and they include mouse [3], *Caenorhabditis elegans* [19], yeast [20], and *Drosophila* [21–28]. Each of these models recapitulates particular aspects of *tau*-associated pathologies [3]. In *Drosophila*, overexpression of human *tau* transgenes in the eye caused disruption of external eye structure [21,23,24,26]. Tau protein was phosphorylated in *Drosophila* [21–23]; and various Tau kinases including Cdk5, GSK3β, c-jun N-terminal kinase, partition defective-1 (PAR-1), and protein kinase A (PKA) had been implicated in *tau*-induced toxicity [21–24]. Although overexpression of *tau* per se did not result in NFT formation [21,23,26], fibrillar aggregates were observed when human Tau protein was coexpressed with the fly GSK3β, Shaggy [21]. In this report, we coexpressed human *tau* transgenes with *GSK3β/Shaggy* or *Cdk5* in the *Drosophila* eye to investigate the relationship between Tau protein hyperphosphorylation, solubility properties, and *tau*-induced toxicity.

Materials and methods

Drosophila genetics. Flies were raised at 25 °C on standard corn meal medium supplemented with dry yeast. Transgenic fly lines carrying *UAS-human wt Tau* (*WT-2*), *UAS-human R406W Tau* (*R406W-1*), and *UAS-human V337M Tau* inserts were gifts of Dr. Mel Feany (Harvard Medical School, USA); *UAS-Cdk5* and *UAS-P35* lines were gifts of Dr. Edward Giniger (Fred Hutchinson Cancer Research Center, USA); *UAS-wt GSK3β/Shaggy*, *UAS-A81T GSK3β/Shaggy*, *gmr-GAL4*, and *vestigial-GAL4* (*vg-GAL4*) were obtained from Bloomington *Drosophila* Stock Center, USA.

Immunoblot analysis. Protein extraction and Western blotting were performed according to [29]. Primary antibodies used include phospho-independent Tau antibody (anti-Total Tau; DAKO; 1:4000), phospho-dependent Tau antibodies: AT-8 (specific for the phosphoserine 202 epitope; Pierce; 1:400); AT-180 (specific for the phosphoserine 231 epitope; Pierce; 1:400), AT-270 (specific for the phosphothreonine 181 epitope; Pierce; 1:400), and anti-β-tubulin antibody (E7; 1:1000; Developmental Studies Hybridoma Bank, University of Iowa, USA).

Calf intestinal phosphatase treatment of protein samples. Calf intestinal phosphatase (400 U/mL; Promega) was added, and samples were incubated at 37 °C for 48 h. The reaction was stopped by the addition of 20 μL SDS sample buffer. The samples were boiled at 100 °C for 2 min and then subjected to SDS–PAGE and immunoblotting as described.

ELISAs. Proteins were extracted from 16 adult fly heads of appropriate genotypes in 75 μL of 1× PBS supplemented with protease inhibitors cocktail (50 μL/mL; Sigma). The Human Total Tau ELISA Kit (BioSource) was used to determine total Tau protein levels in fly head samples. The ELISAs were performed according to manufacturer's instructions.

Semi-quantitative RT-PCRs. Total RNA was extracted from adult fly heads using Trizol reagent (Invitrogen) according to [30]. To study the mRNA expression level of *GSK3β/Shaggy* in flies, RT-PCR was performed and *actin* was used as internal control. PCR conditions were as follows: 95 °C for 6 min; followed by 95 °C for 30 s, 68 °C for 30 s, and 75 °C for 30 s for 35 cycles. *GSK3β/Shaggy* primers used were: 5′ CGT ATA CGC ACT CCT ACA GAA G 3′ and 5′ GGC CGC AAT GGA GGC TGC TC 3′; and *actin* primers used were: 5′ ATG TGC AAG GCC GGT TTC GC 3′, and 5′ CGA CAC GCA GCT CAT TGT AG 3′. RT-PCR results generated from three independent sets of RNA preparations were used for analysis.

Sarcosyl extraction of fly head proteins. Thirty fly heads were homogenized in 40 μL Tris-buffered saline (10 mM Tris, pH 7.4, 0.8 M NaCl, 1 mM EGTA, 10% sucrose, and 50 μL/mL protease inhibitors cocktail). The homogenates were then centrifuged at 20,000*g* at 4 °C for 30 min. The supernatants were retained, and pellets were resuspended in 20 μL of Tris-buffered saline and re-centrifuged. Supernatants were combined and brought to 1% *N*-lauroylsarcosinate (w/v). Samples were incubated at room temperature for 1 h with shaking, followed by centrifugation at 100,000*g* at room temperature for 1 h. The supernatants were then collected as the sarcosyl-soluble fraction. The pellets were resuspended in 20 μL 50 mM Tris–HCl, pH 7.4, to form the sarcosyl-insoluble fraction. Finally, SDS sample buffer was added to both fractions to a final volume of 60 μL.

Sequential extraction of fly head proteins. Two sequential extraction protocols were used. First, 30 adult fly heads were homogenized in 40 μL of high-salt buffer (HSB; 50 mM Tris, pH 7.4, 750 mM NaCl, 5 mM EDTA, pH 8, and 50 μL/mL protease inhibitors cocktail) and centrifuged at 434,000*g* at 4 °C for 30 min. The pellets were re-extracted with 15 μL of fresh HSB, and supernatants were pooled and regarded as HS fraction. The pellets were then extracted twice (25 μL each) with HSB supplemented with 1% Triton X-100. The supernatants were pooled and regarded as HS/Triton X-100 (HST) fraction. Subsequently 60 μL of SDS sample buffer was added to resuspend the HST-insoluble pellets, which formed the SDS fraction. For the second protocol, 30 adult fly heads were homogenized in 40 μL HSB and centrifuged at 434,000*g* at 4 °C for 30 min. The pellets were re-extracted with 15 μL of fresh HSB, and supernatants were then pooled and regarded as HS fraction. The pellets were then extracted twice (25 μL each) with 2% SDS. The supernatants were pooled and regarded as SDS fraction. The SDS-insoluble pellets were incubated with 30 μL of 70% formic acid (FA) at 37 °C for 30 min. The supernatants were collected as 70% FA fraction after centrifugation at 434,000*g* at 4 °C for 30 min. The resulting pellets were then incubated with 30 μL of 100% FA at 37 °C for 30 min to form the 100% FA fraction. FA was removed by Speed-Vac centrifugation and 60 μL SDS buffer was then added to the samples.

Histology and immunohistochemistry. For scanning electron microscopy, fly heads were fixed in 2.5% glutaraldehyde (EM grade, Electron Microscopy Sciences) in phosphate buffer for 4 h, post-fixed with 1% osmium tetroxide (Electron Microscopy Sciences), dehydrated to 100% ethanol, and critical-point dried with liquid CO_2. Gold-palladium-coated specimens were examined with a Jeol JSM-6301FE microscope operated at 5 kV. For cryosectioning, adult fly heads were embedded in OCT (Tissue-Tek) at −20 °C, cut into 12 μm sections. Primary antibodies used were anti-Total Tau (DAKO; 1:100) and AT-100 (specific for phosphoserine 212 and phosphothreonine 214 epitopes; Pierce; 1:150).

Lithium treatment. Two different protocols were used to administer lithium chloride (LiCl) to *Drosophila*. First, newly enclosed flies were raised on normal fly food for 2–3 days. Flies were then starved for 4 h and subsequently allowed to feed on 5% glucose solution supplemented with 80, 160 or 240 mM of LiCl at 25 °C for 16 h. NaCl was used as control. This protocol was adopted for the examination of the effect of Li on Tau protein phosphorylation. For the second protocol, parental flies were raised on fly food containing 2 mM of LiCl at 25 °C, and progeny was collected 2–3 days after eclosion for analysis. The second protocol was adopted to investigate the effect of Li on the *vg-GAL4 + GSK3β/Shaggy* wingless phenotype. After LiCl treatment, adult fly heads were homogenized in SDS sample buffer for atomic absorption spectrometry (Varian, Atomic Absorption Spectrometer 640) to determine Li intake.

Characterization of the vestigial-GAL4 GSK3β/Shaggy-induced wingless phenotype. A wingless phenotype was observed when *GSK3β/Shaggy* was overexpressed with the *vg-GAL4* promoter. Based on the severity of this wingless phenotype (Fig. 5c), a six-level progressive scoring scheme was established to access the kinase activity of GSK3β/Shaggy. Level 1 was defined as flies bearing normal wild-type wing structure, while level 6 was defined as flies that exhibit wingless phenotype. An average of 50–100 flies were scored for each experiment.

Statistical analysis. Unpaired *t* test was performed to detect statistical differences among experimental groups. A significance of difference was determined at the $p < 0.05$ level.

Results

Transgenic overexpression of human Tau protein is hyperphosphorylated and toxic in Drosophila

We showed that overexpression of Tau protein, including wild-type (wt), R406W, and V337M, driven by *gmr-GAL4* was toxic and caused dominant rough eye phenotype (Fig. 1a). Multiple protein bands were detected by the Total Tau antibody, indicating that transgenic human Tau protein underwent various degrees of phosphorylation (Fig. 1b). We did not observe any alteration of transgenic Tau protein level when *tau* transgenes were overexpressed alone or coexpressed with Tau protein kinase (Fig. 1c). Further, phosphorylation of individual tauopathies-related epitopes of the overexpressed transgenic human Tau protein was also detected with phospho-dependent Tau antibodies (Fig. 1b).

When *GSK3β/Shaggy* was coexpressed with wt *tau*, a single protein band with reduced electrophoretic mobility was detected using the Total Tau antibody (Fig. 1c, lane 1). Tau protein hyperphosphorylation was also observed with phospho-dependent antibodies, including AT-270 (Fig. 1c). When the wt *tau*-expressing fly head samples were treated with calf intestinal phosphatase in vitro, all Tau proteins migrated as a single protein band of around 50 kDa which corresponded to the predicted size of Tau without any posttranslational modification (Fig. 1c). Altogether, our data show that overexpressed human Tau protein can be hyperphosphorylated by the coexpression of protein kinase in *Drosophila*. Similar results were also observed in R406W and V337M Tau (data not shown).

Overexpression of GSK3β/Shaggy and Cdk5 causes Tau hyperphosphorylation and exacerbates tau-induced toxicity

We showed that *GSK3β/Shaggy* coexpression intensified *tau*-induced toxicity as indicated by enhanced deterioration of the external eye morphology (Fig. 2a). Such enhancement was also associated with concomitant hyperphosphorylation of Tau (Fig. 2b). Next, we coexpressed *wt tau* with a mutant kinase [31] transgene *A81T GSK3β/Shaggy* to investigate whether kinase activity of GSK3β/Shaggy was crucial for Tau hyperphosphorylation and its

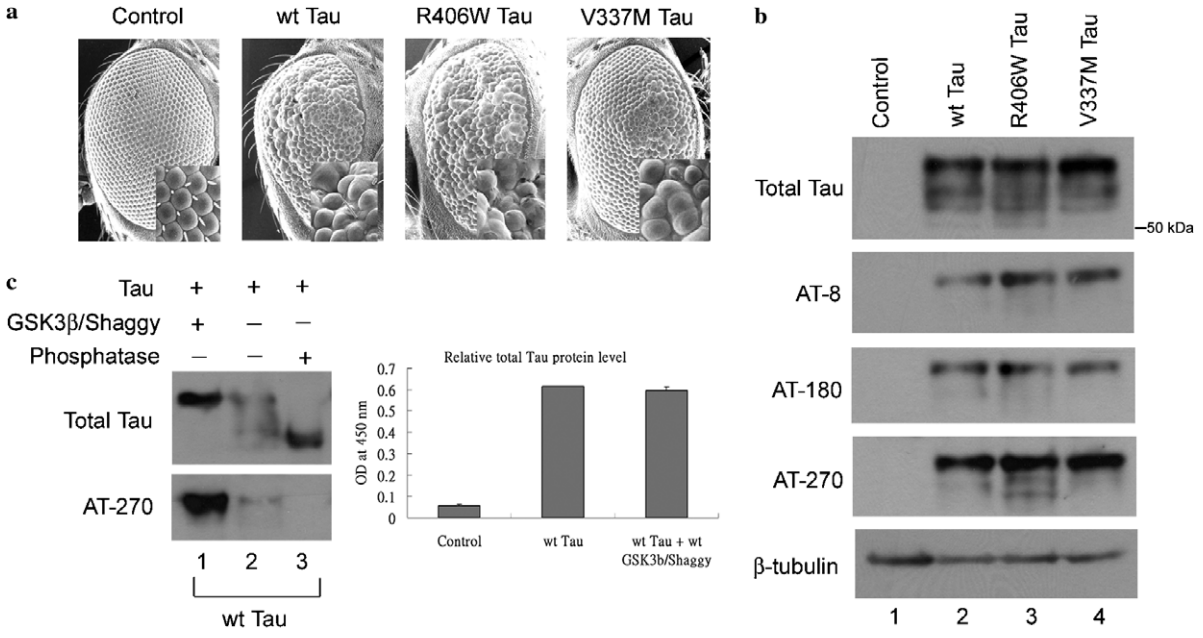

Fig. 1. Overexpression of human Tau protein in adult *Drosophila* eye produced a rough eye phenotype and enhanced Tau protein phosphorylation. (a) Flies overexpressing wild-type (wt), R406W, and V337M Tau proteins developed rough eye phenotype with disorganized ommatidial structure; while flies bearing *gmr-GAL4* alone showed normal external eye morphology. (b) All human *tau* transgenes (lanes 2–4) were expressed (Total Tau panel) and phosphorylated (AT-8, AT-180, and AT-270 panels) in *Drosophila*. No Tau protein was detected in flies carrying no human *tau* transgene (lane 1); this confirmed the specificity of the antibodies used. (c) Transgenic wild-type (wt) Tau protein was phosphorylated by endogenous protein kinases (lane 2). When the *tau* transgenes were coexpressed with *GSK3β/Shaggy*, electrophoretic mobility of Tau protein was reduced in the Total Tau panel (lane 1), and the intensity of the Tau band increased in the AT-270 panel (lane 1). These indicate hyperphosphorylation of Tau protein. Calf intestinal phosphatase (CIP) treatment resulted in an increase in the electrophoretic mobility of Tau (Total Tau panel; lane 3), indicating the removal of phosphate groups on the Tau protein. Dephosphorylation of Tau was further confirmed by the disappearance of the phospho-dependent Tau band recognized by the AT-270 antibody (AT-270 panel; lane 3). "+" lanes represent CIP treatment, whereas "–" lanes represent mock treatment. β-Tubulin was used as internal loading control. Results shown are representatives of at least three independent experiments. ELISA showed that either in the presence or absence of *GSK3β/Shaggy* coexpression, wt Tau protein levels were similar. Flies of genotypes *w*; *gmr-GAL4*/+; +/+ (control), *w*; *gmr-GAL4*/+; *UAS-wt-Tau*/+ and *w*; *gmr-GAL4*/+; *UAS-R406W Tau*/+ and *UAS-V337M Tau*/*w*; *gmr-GAL4*/+; +/+.

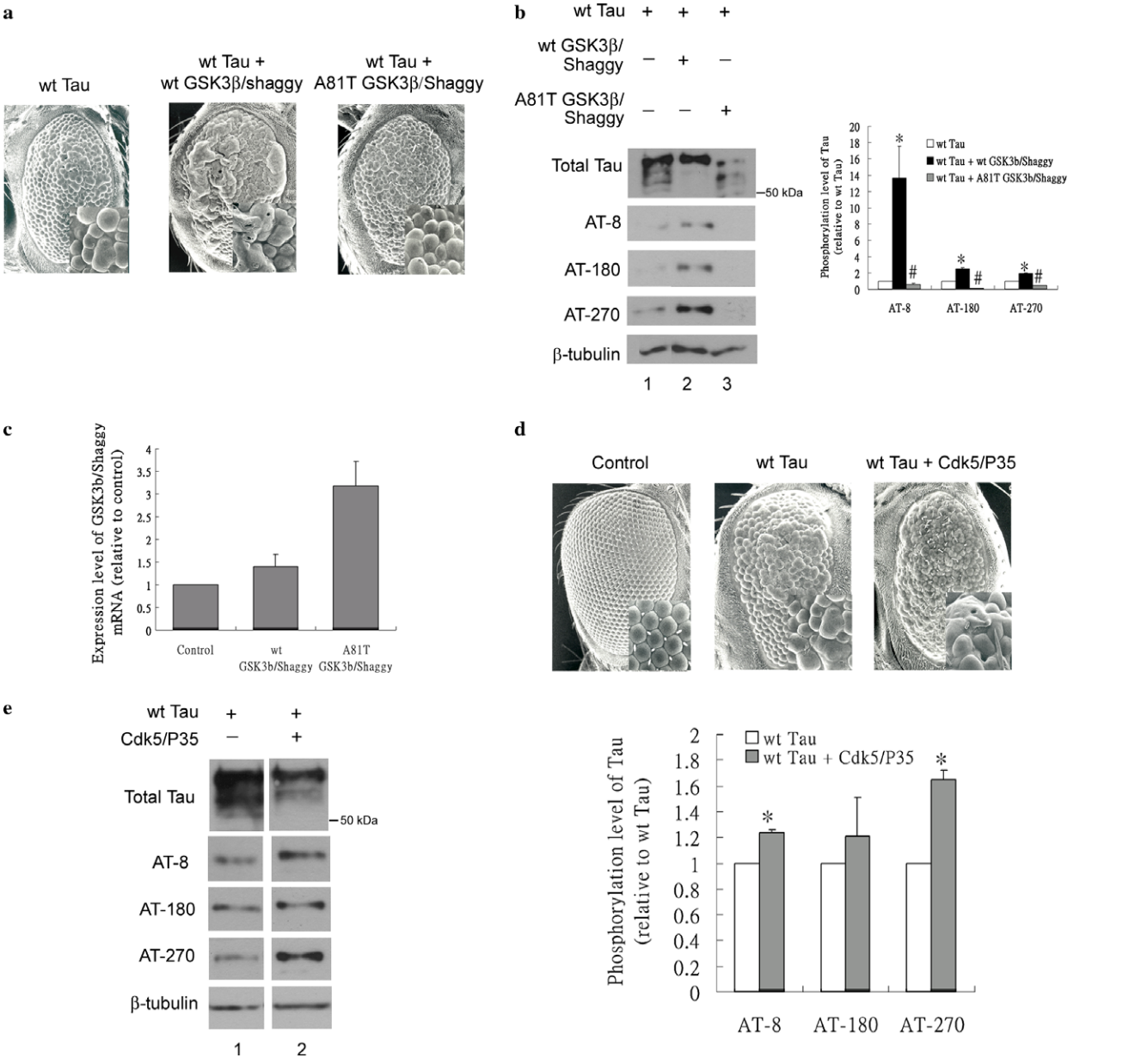

Fig. 2. (a) Coexpression of *tau* and *wt GSK3β/Shaggy* enhanced *tau*-induced rough eye phenotype. However, flies coexpressing *wt tau* and *A81T GSK3β/Shaggy* mutant showed a comparable rough eye phenotype as of flies overexpressing the *tau* transgene alone. (b) Electrophoretic mobility of Tau protein in *wt tau* + *A81T GSK3β/Shaggy* expressing flies (Total Tau panel; lane 3) was comparable to that of flies overexpressing *wt tau* alone (Total Tau panel; lane 1). Similarly, flies coexpressing *wt tau* and *A81T GSK3β/Shaggy* mutant failed to hyperphosphorylate Tau at particular phospho-specific epitopes (AT-8, AT-180, and AT-270 panels; lane 3). The phosphorylation levels of different Tau epitopes were expressed as fold change compared to the *wt Tau* alone control. Results are plotted as means with SEM of three independent experiments. Phosphorylation levels of Tau were significantly higher in *wt tau* + *wt GSK3β/Shaggy*-coexpressing flies than that of the *wt tau* alone control ($^{*}p < 0.05$). Coexpression of *wt tau* with *A81T GSK3β/Shaggy* mutant resulted in a significantly lower level of phosphorylation when compared to flies coexpressing *wt tau* and *wt GSK3β/Shaggy* ($^{\#}p < 0.05$). (c) mRNA expression of *A81T GSK3β/Shaggy* mutant transgene was higher than that of the *wt GSK3β/Shaggy* transgene. Data were obtained from three independent sets of experiments, and results are expressed as means with SEM of three independent experiments. (d) Flies coexpressed with *wt tau* and *Cdk5/P35* showed a more severe rough eye phenotype and a reduction of eye size, when compared to flies overexpressing *wt tau* alone. (e) A mild reduction of electrophoretic mobility of Tau protein was observed when *Cdk5/P35* were coexpressed with *wt tau* (Total Tau panel). A significant increase of Tau phosphorylation levels at specific Tau epitopes, AT-8 and AT-270, was also observed ($^{*}p < 0.001$). Results are expressed as means with SEM of three independent experiments. Tau protein phosphorylation was analyzed and expressed as fold change of the *wt Tau* alone control. β-Tubulin was used as internal loading control. Flies of genotypes *w*; *gmr-GAL4/+*; *+/+* (control; c and d), *w*; *gmr-GAL4/+*; *UAS-wt-Tau/+* (a, b, d, and e), *w*; *gmr-GAL4/UAS-wt-GSK3β/Shaggy*; *UAS-wt-Tau/+* (a,b), *w*; *gmr-GAL4/+*; *UAS-wt-Tau/UAS-A81T-GSK3β/Shaggy* (a,b), and *w*; *gmr-GAL4/UAS-Cdk5*; *UAS-wt-Tau/UAS-P35* (d,e).

phenotypic enhancement. Unlike *wt GSK3β/Shaggy*, hyperphosphorylation of Tau was not observed with the *A81T GSK3β/Shaggy* mutant, and a phosphorylation pattern similar to that of sole expression of *wt tau* was observed (Fig. 2b, lanes 1 and 3). Consistently, no enhancement of the *tau*-induced rough eye phenotype was observed

when *wt tau* was coexpressed with the *A81T GSK3β/Shaggy* mutant transgene (Fig. 2a). Our data thus confirmed that kinase activity of GSK3β/Shaggy is crucial for Tau protein hyperphosphorylation and *tau*-induced toxicity. Similar results were also obtained with the R406W and V337M mutant Tau proteins (data not shown).

Cyclin-dependent kinase 5 (Cdk5) is another protein kinase that has been implicated in Tau protein phosphorylation in vivo [32]. Cdk5 becomes active when associates with its neuronal-specific co-activator P35 [33]. *Cdk5/P35* coexpression enhanced the *tau*-induced rough eye phenotype as indicated by the reduction of eye size and more severe deterioration of external eye structure (Fig. 2d). Although Cdk5/P35 caused Tau protein hyperphosphorylation in flies, no significant hyperphosphorylation of the AT-180 epitope was detected when compared to other phospho-dependent epitopes examined (Fig. 2e).

Tau protein hyperphosphorylation alters its solubility properties and enhances tau-induced toxicity

Alteration of protein solubility properties has been shown to associate with pathologic potentials of Tau [34,35], we therefore investigated whether protein hyperphosphorylation altered protein solubility properties of Tau in flies. Sarcosyl fractionation is a widely used method to determine solubility of Tau protein [36–38]. We found that majority of Tau protein could be solubilized in 1% sarcosyl, and only a minimal amount of sarcosyl-insoluble Tau was detected in 2-day-old flies when *GSK3β/Shaggy* was coexpressed (Fig. 3a, lanes 1–4).

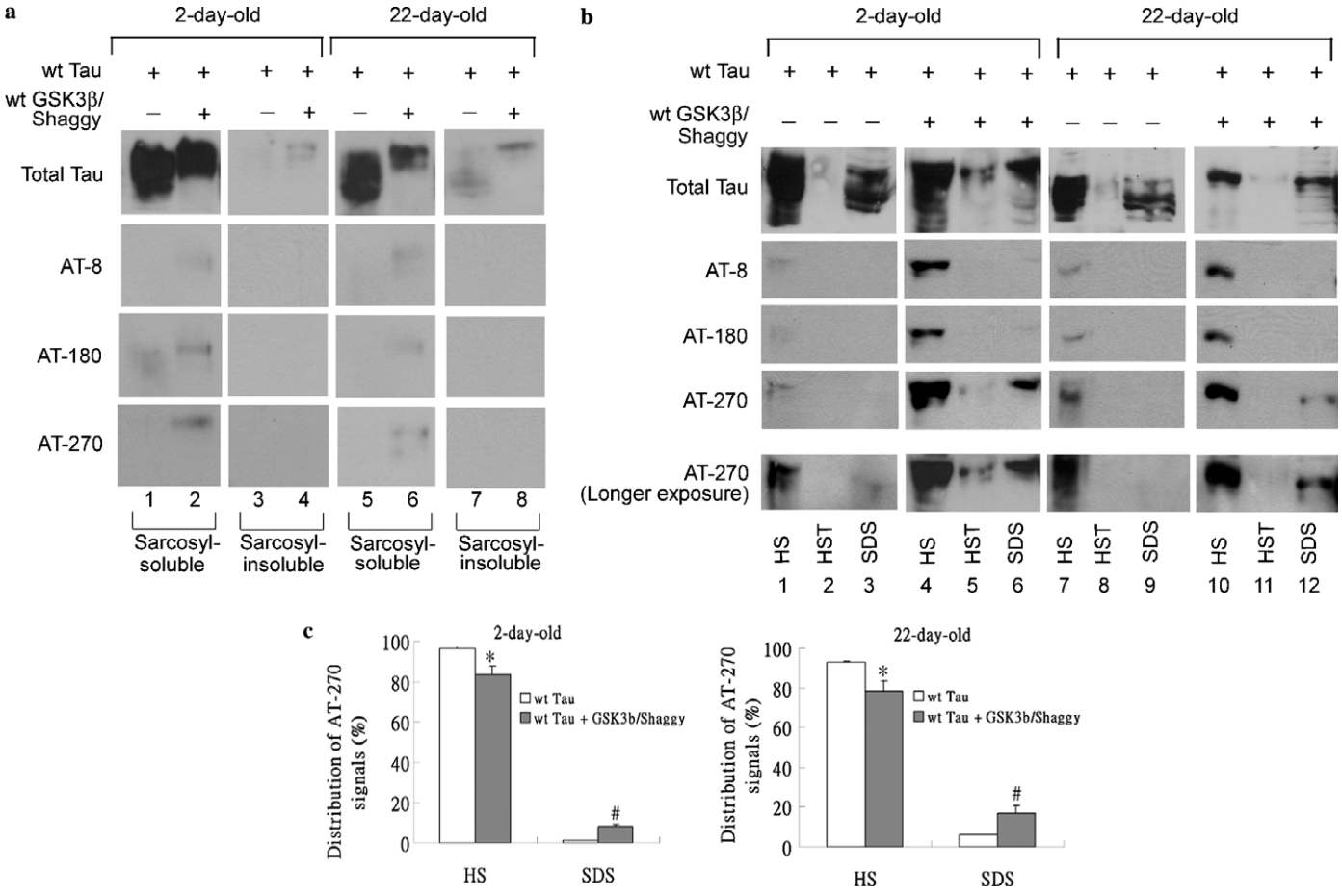

Fig. 3. Sarcosyl- and sequential extraction of Tau protein. (a) Upon sarcosyl treatment most Tau protein was extracted and partitioned in the sarcosyl-soluble fraction in both 2- and 22-day-old flies overexpressed with the *wt tau* transgene, regardless of their phosphorylation status (lanes 1, 2, 5, and 6). Only residual amount of Tau protein was detected in the sarcosyl-insoluble fraction (Total Tau panel; lanes 3, 4, 7, and 8). Apart from protein hyperphosphorylation (reduction of electrophoretic mobility of Tau protein bands as detected by phospho-independent antibody), *GSK3β/Shaggy* coexpression did not alter the distribution of Tau protein among sarcosyl-soluble and -insoluble fractions. (b) Tau protein was sequentially extracted with buffers of increasing protein-solubilizing ability (high-salt (HS), HS/Triton X-100 (HST), and SDS). Results shown are representatives of three independent experiments. In both 2- and 22-day-old flies, most Tau proteins were solubilized in the HS- (lanes 1, 4, 7, and 10) and SDS-soluble (lanes 3, 6, 9, and 12) fractions. Alteration of the distribution of the HS-soluble and SDS-soluble Tau was observed when *GSK3β/Shaggy* was coexpressed with *wt tau* (lanes 4, 6, 10, and 12) when compared to the *wt tau* alone control. (c) The distribution of the AT-270 Tau signals among HS-soluble and SDS-soluble fractions was analyzed. Results are plotted as percentage of Total Tau protein intensity of all fractions (HS, HST, and SDS), and expressed as means with SEM of three independent experiments. The percentage of HS-soluble Tau signal was significantly lower in flies coexpressing *wt tau* and *GSK3β/Shaggy* when compared to that of the *wt tau* alone control ($^{*}p < 0.05$). This was also accompanied with a significant increase in the percentage of SDS-soluble Tau fraction ($^{\#}p < 0.05$). Flies of genotypes *w*; *gmr-GAL4/+*; *UAS-wt-Tau/+*, *w*; *gmr-GAL4/UAS-wt-GSK3β/Shaggy*; *UAS-wt-Tau/+*.

We next performed sequential protein extraction to further investigate the solubility properties of Tau in detail [36]. Results from 2-day-old flies showed that a large proportion of Tau protein was soluble in 750 mM NaCl (high-salt buffer; HSB) and 2% SDS (Fig. 3b, lanes 1 and 3), and only low level of Tau was partitioned in the HS/Triton X-100 (HST) fraction (Fig. 3b, lane 2). This clearly indicates that two species of Tau with distinct solubility properties (HS-soluble and SDS-soluble) were present in flies. To rule out the possibility that SDS-insoluble Tau was present in the protein samples, we extracted proteins sequentially with formic acid in an attempt to retrieve any Tau in the SDS-insoluble pellets. Formic acid has widely been used to dissolve aggregated Tau protein [15,34,35,39]. Only residual amount of Tau was extracted in 70% and 100% formic acid (data not shown). This is coherent with the sarcosyl extraction data that most Tau proteins are detergent-soluble (Fig. 3a).

We showed that the AT-8, AT-180, and AT-270 antibodies detected mainly the HS-soluble Tau species (Fig. 3b, lane 1), and the intensity of the signals increased when the human *tau* transgene was coexpressed with *GSK3β/Shaggy* (Fig. 3b, lane 4). Interestingly, we detected a novel SDS-soluble Tau species with the AT-270 antibody in *tau* flies coexpressed with *GSK3β/Shaggy* (Fig. 3b, lane 6). This indicates that there is a change in Tau protein solubility properties in these flies (Fig. 3b, AT-270 panel, lanes 3 and 6). Further, we detected a shift of distribution of the AT-270 signals from the HS- to SDS-soluble fractions in *tau* + *GSK3β/Shaggy* coexpressed flies. There was a significant increase of the SDS-soluble Tau species together with a concomitant decrease in HS-soluble Tau species when *wt tau* was coexpressed with *GSK3β/Shaggy* (Fig. 3c). However, no significant alteration of HST-soluble Tau species between *tau* and *tau* + *GSK3β/Shaggy* was observed (data not shown). Our data therefore suggest that the ratio of HS-soluble to SDS-soluble Tau is tightly linked to *tau*-induced toxicity. We also observed similar trends when R406W and V337M *tau* were coexpressed with *GSK3β/Shaggy* or *Cdk5/P35* (data not shown). Altogether, our results indicate that Tau protein hyperphosphorylation correlates with (1) alteration of Tau protein solubility properties and (2) the enhancement of *tau*-induced toxicity.

Tau-positive aggregates are detected in aged transgenic flies

Since we detected low level of sarcosyl-insoluble Tau biochemically in *tau* transgenic fly lines (Fig. 3a), we performed immunostaining with both phospho-independent and -dependent Tau antibodies to check if PHF-like aggregate structures were present in these flies. Consistent with previous findings [23,26], we did not detect any Tau-positive aggregate in 2-day-old flies even when *GSK3β/Shaggy* was coexpressed (data not shown). In aged flies (22-day-old), Tau-positive Tau aggregates were observed

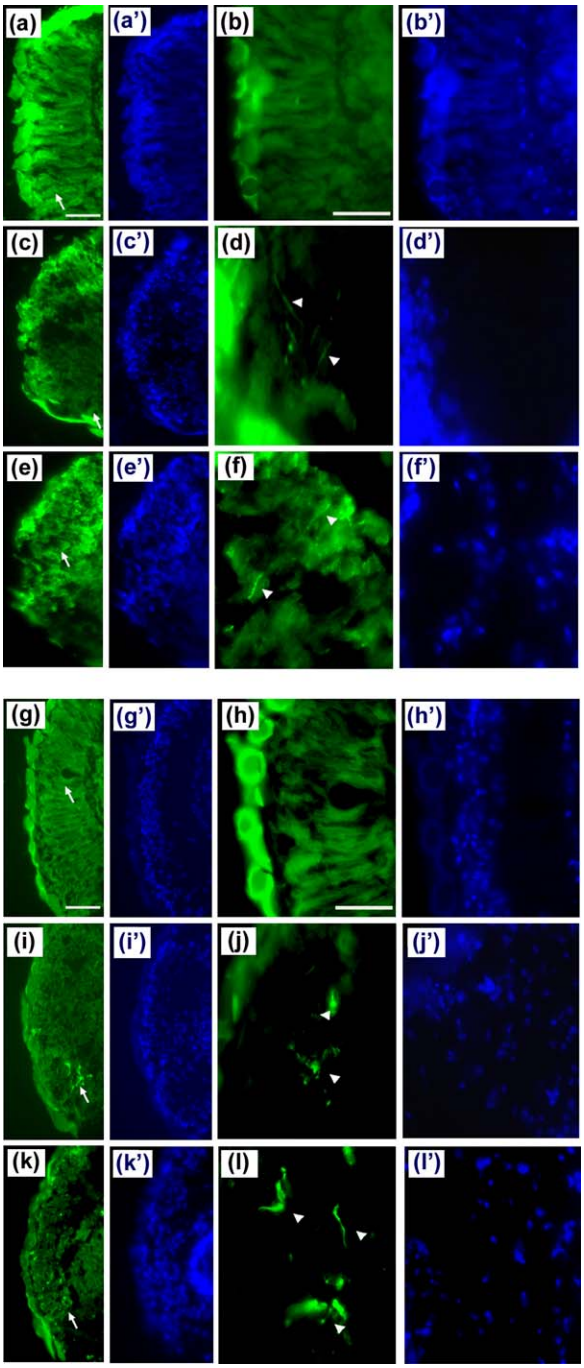

Fig. 4. Aged flies overexpressing human *tau* transgenes developed Tau-positive aggregates. (a–d) Adult head sections of 22-day-old were immunostained with "Total Tau" (a–f) and AT-100 antibodies (g–l). DAPI was used to label cell nuclei (a′–l′). Control flies (*gmr-GAL4*) showed an organized retinal structure, and no Tau-immunoreactive aggregate was detected (a, b, g, and h). (c–f and i–l) Tau-immunoreactive aggregates (arrowheads) were observed in flies overexpressed with either *wt tau* alone (c, d, i, and j) or coexpressed with *GSK3β/Shaggy* (e, f, k, and l). Disorganization of retina was also observed in these flies (c–f and i–l). Results shown are representative of at least three independent experiments. Arrows show the magnified areas shown in (b, d, f, h, j, and l). Flies of genotypes *w*; *gmr-GAL4*/+; +/+ (control), *w*; *gmr-GAL4*/+; *UAS-wt-Tau*/+, *w*; *gmr-GAL4*/*UAS-wt-GSK3β*/*Shaggy*; *UAS-wt-Tau*/+. Scale bars represent 20 μm.

(Fig. 4). These aggregates were found to be distributed in the retina of *tau*-expressing flies with (Fig. 4e, f, k, and l) or without (Fig. 4c, d, i, and j) *GSK3β/Shaggy* coexpression. This indicates that aging is one contributing factor to Tau-positive aggregate formation. Our data are also consistent with previous findings that Tau hyperphosphorylation per se does not induce Tau protein aggregation [23].

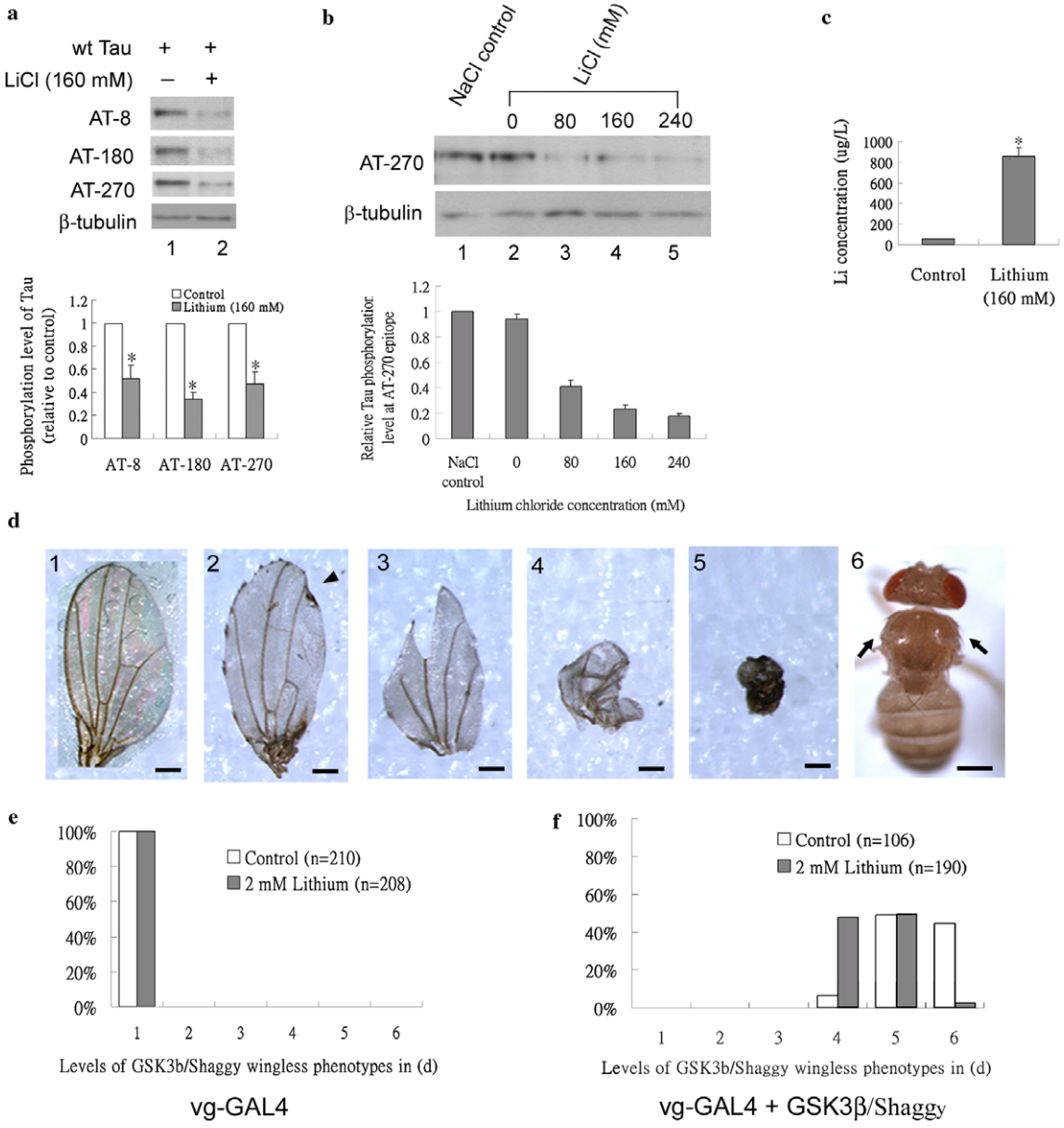

Fig. 5. The effect of lithium on GSK3β/Shaggy kinase activity in *Drosophila*. (a) Upon LiCl treatment, reduction of phospho-dependent Tau epitopes was observed (AT-8, AT-180, and AT-270 panels). The phosphorylation levels of different Tau epitopes were expressed as fractions of that of control flies (*wt tau* alone), and results are plotted as means with SEM of three independent experiments. Phosphorylation level of Tau epitopes was significantly lower in flies fed with LiCl ($*p < 0.01$). β-Tubulin was used as loading control. (b) Dose–response of LiCl inhibition of Tau protein phosphorylation. The phosphorylation levels of AT-270 Tau epitope were expressed as fractions of that of control flies (NaCl control), and results are plotted as means with SEM of three independent experiments. β-Tubulin was used as loading control. (c) A significant increase of cellular Li concentration was observed in flies treated with 160 mM LiCl when compared to those without treatment ($*p < 0.01$). Data were obtained from three independent sets of experiments, and results are expressed as means with SEM. (d) A wingless phenotype was observed when *wt GSK3β/Shaggy* transgene was overexpressed by the *vg-GAL4* promoter. The severity of the *vg-GAL4* + *GSK3β/Shaggy* wingless phenotype was classified into six levels, and such scale was used to estimate GSK3β/Shaggy kinase activity. Level 1 is defined as flies with wild-type wing morphology, level 2 as normal wings but with broken wing margin (arrowhead); level 3 as wings that are of ~80% of normal size; level 4 as wings that are of ~50% of normal size; level 5 as wings that are of ~10% of normal size, and level 6 is the most severe category that flies are wingless (arrows). (e) Flies without *GSK3β/Shaggy* overexpression all displayed wild-type wing morphology (level 1). (f) The percentage of wingless flies (level 6) was reduced from 45% to less than 3% in *vg-GAL4* + *GSK3β/Shaggy* flies treated with LiCl. Similar trends were also observed in another independent experiment (Supplementary Fig. 3). Flies of genotype *w*; *vg-GAL4*/+; *UAS-wt Tau*/+ (a,b), *w*; *vg-GAL4*/+; +/+ (c,e), and *w*; *vg-GAL4*/*UAS-wt-GSK3β*/*Shaggy*; +/+ (c,e). Scale bars represent 100 μm (d1–5), 1 mm (d6).

Lithium administration reduces Tau phosphorylation but cannot mitigate tau-induced toxicity

Lithium chloride (LiCl) inhibits GSK3β/Shaggy kinase activity [40–42]. Here, we investigated the effects of Li on Tau phosphorylation status in adult flies. Levels of Tau protein phosphorylation were significantly reduced when adult flies were treated with 160 mM LiCl for 16 h (Fig. 5a), and we also observed a dose-dependent inhibition of Tau protein phosphorylation (Fig. 5b). However, we were unable to detect any morphological alteration of the *tau*-induced rough eye phenotype even when we initiated LiCl treatment from the third instar larval stage (data not shown). To correlate Tau protein dephosphorylation with Li intake in adult flies, we quantified the cellular Li ion concentration by atomic absorption spectrometry, and found a significant increase of cellular Li concentration in flies that had been fed with LiCl (Fig. 5c).

To further investigate the effects of Li on GSK3β/Shaggy kinase activity, we overexpressed *GSK3β/Shaggy* in the *Drosophila* wing (Fig. 5d). When *GSK3β/Shaggy* was overexpressed in the fly wing using *vestigial-GAL4* (*vg-GAL4*), a wingless phenotype was observed (Fig. 5d). We characterized the *vg-GAL4 + GSK3β/Shaggy*-induced wingless phenotype by categorizing the phenotype, according to its severity, into six levels (Fig. 5d). When control flies (*vg-GAL4* alone) were treated with LiCl, no alteration of wing morphology was observed (Fig. 5e). This suggests that Li exerts no observable deleterious effect on normal wing development. Upon LiCl treatment, the severity of the *vg-GAL4 + GSK3β/Shaggy*-induced wing phenotype was reduced (Fig. 5f). The percentage of "level 6" wingless flies decreased from 45% to 3%, which was accompanied with a concomitant increase in the number of flies with the less severe "level 4" wing phenotype (from 7% to 48%, Fig. 5f). Similar trends were also observed in another independent trial (Supplementary Fig. 3). Apart from confirming the effect of Li on inhibiting GSK3β/Shaggy kinase activity, our results also illustrate that the *vg-GAL4 + GSK3β/Shaggy*-induced wing phenotype can be used as a simple but robust in vivo model for the screening of GSK3β/Shaggy inhibitors.

Discussion

In the present study, we performed detailed biochemical analyses to investigate the effects of Tau protein phosphorylation on *tau*-induced toxicity.

Pre-tangle events

Consistent with a previous report [23], we showed that the enhancement of *tau*-induced toxicity is not accompanied with Tau-positive aggregate formation in young flies (2-day-old, Figs. 2 and 4). Although it has been observed in a separate report that the enhancement of the *tau*-induced rough eye phenotype was accompanied with Tau aggregate formation [21], this could be explained by the difference in transgene expression levels. For example, it had been reported that high levels of transgenic Tau protein expression caused filamentous inclusions formation, whereas relatively lower transgene expression levels only resulted in pre-tangle neurons [43]. The data presented here further support the notion that hyperphosphorylated, non-aggregated Tau protein is toxic [23], and that *Drosophila* can be used to study pre-tangle events of Tau pathologies [3].

Tau protein solubility properties and toxicity

In our model, most Tau protein was sarcosyl-soluble, and only a low level of sarcosyl-insoluble Tau was detected even when *GSK3β/Shaggy* was coexpressed (Fig. 3a). We did not find any correlation between formation of sarcosyl-insoluble Tau protein (Fig. 3a) and *GSK3β/Shaggy* enhancement of *tau*-induced toxicity (Fig. 2a). On the other hand, with the use of a phospho-dependent antibody AT-270 [44], we detected an alteration of Tau protein solubility properties when *GSK3β/Shaggy* was coexpressed (Fig. 3b). In both young (2-day-old) and aged (22-day-old) flies, we observed significant increase in the amount of SDS-soluble Tau and a concomitant decrease in HS-soluble Tau species in *GSK3β/Shaggy* coexpressed flies (Fig. 3c). Although the SDS-soluble AT-270 Tau signal in 22-day-old flies was significantly higher than that of 2-day-old *GSK3β/Shaggy* coexpressed flies, no further enhancement of *tau*-induced rough eye phenotype was observed. This may be due to the fact that *GSK3β/Shaggy* enhancement of *tau*-induced rough eye phenotype had already reached its maximum 2 days after eclosion, and therefore no further external eye deterioration could be observed. Nevertheless, our data would still indicate that hyperphosphorylation causes alteration of Tau protein solubility properties, which in turn leads to Tau toxicity (Figs. 2a and 3b). Therefore, our data suggest that the AT-270 epitope of Tau would be a key determinant for the enhancement of *tau*-induced toxicity (Fig. 3b). Our findings are also coherent with previous reports that detergent-soluble hyperphosphorylated forms of Tau are toxic [23,38].

Tau aggregates and aging

By immunoblotting, small amount of sarcosyl-insoluble Tau in young flies (2-day-old, Fig. 3a) was detected which is indicative of the presence of PHF-Tau. Our data are consistent with other previous reports [23,26] that no Tau-positive aggregates were detected by immunohistochemistry in young transgenic flies (2-day-old). Although we detected sarcosyl-insoluble Tau in both young (2-day-old) and aged (22-day-old) flies (Fig. 3a and b), we only observed Tau-positive aggregates in aged flies regardless of *GSK3β/Shaggy* coexpression (Fig. 4). Our data therefore indicate that the formation of Tau-positive aggregates was not related to the alteration of *tau*-induced toxicity in our model.

Our results further suggest that additional factors, such as alteration of protein conformation [45], which might have happened during the aging process may trigger Tau aggregate formation.

Pharmacological intervention of Tau phosphorylation and GSK3β/Shaggy kinase activity

We employed a pharmacological approach (Fig. 5) to validate our *A81T GSK3β/Shaggy* mutant analysis results (Fig. 2). Lithium is a characterized inhibitor of GSK3β/Shaggy [8]. In mice, Li administration had been shown to reduce Tau phosphorylation and PHF formation [41,42]. In the present study, we showed that feeding adult flies with LiCl inhibited Tau protein phosphorylation (Fig. 5a), and that LiCl treatment did not alter Tau protein expression (Supplementary Fig. 2). We further demonstrated the effect of Li on the inhibition of Tau protein phosphorylation was associated with an increase in cellular Li intake (Fig. 5c). However, no phenotypic modification of the adult *tau*-induced rough eye phenotype was observed despite the fact that Tau protein phosphorylation was reduced. This could be due to the high levels of prolonged expression of the *tau* transgene.

In an attempt to investigate the effects of Li on GSK3β/Shaggy kinase activity in *Drosophila*, we overexpressed *GSK3β/Shaggy* in the fly wing (Fig. 5d). The *Drosophila* wing had previously been used successfully to demonstrate the effects of γ-secretase inhibitors [46]. The GSK3β/Shaggy kinase antagonizes the wingless signaling pathway. Increase in GSK3β/Shaggy kinase activity causes alteration of gene transcription activity [31], and we showed that overexpression of *GSK3β/Shaggy* in the wing resulted in a wingless phenotype (Fig. 5d). We further demonstrated that LiCl administration is able to modulate the severity of the *vg-GAL4 + GSK3β/Shaggy* wingless phenotype (Fig. 5e–f; Supplementary Fig. 3). Thus, the *vg-GAL4 + GSK3β/Shaggy* phenotype can be developed into an in vivo model to test and/or screen for GSK3β/Shaggy inhibitors.

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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.bbrc.2006.05.112.

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