Biochemical and Biophysical Research Communications 391 (2010) 510–516

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Drosophila notal bristle as a novel assessment tool for pathogenic study of Tau toxicity and screening of therapeutic compounds

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ARTICLE INFO

Article history:
Received 4 November 2009
Available online 18 November 2009

Keywords:
Notum
PP2A
Alsterpaullone
Tauopathy

ABSTRACT

To elucidate the Tau gain-of-toxicity functional mechanism and to search for potential treatments, we overexpressed human Tau variants (hTau) in the dorsal mesothorax (notum) of *Drosophila*. Overexpression of Tau variants caused loss of notal bristles, and the phenotype was used for evaluating toxicity of ectopic Tau. The bristle loss phenotype was found to be highly associated with the toxicity of hyperphosphoryled Tau in flies. We have shown that the bristle loss phenotype can be rescued either by reducing Glycogen synthase kinase 3β (GSK3β)/Shaggy (Sgg) activity or overexpressing Bβ2 regulatory subunits of PP2A. Elevated expression of the *Drosophila* Bβ2 homolog, Twins (Tws), also alleviated neuritic dystrophy of the dorsal arborization (da) neuron caused by Tau aggregation. Additionally, lowering endogenous Tau dosage was beneficial as it ameliorated the bristle loss phenotype. Finally, the bristle loss phenotype was used to evaluate the efficacy of potential therapeutic compounds. The GSK3β inhibitor, alsterpaullone, was found to suppress toxicity of Tau in a concentration-dependent manner. The notum of *Drosophila*, thus, provides a new tool and insights into Tau-induced toxicity. It could also potentially assist in screening new drugs for possible therapeutic intervention.

Introduction

The accumulation of hyperphosphorylated Tau-induced neurofibrillary tangles (NFT) is the primary cause and common pathological hallmark for tauopathy. Since the presence of NFT is highly correlated with the progression, duration and severity of neurodegeneration, studies of its pathogenesis have focused on the molecular and cellular events that lead to the aberrant formation and accumulation of NFT. Tau is reversibly phosphorylated by several kinases and phosphatases. Furthermore, imbalance of kinase and phosphatase activities has been linked to the Tau-associated pathologies (for recent review [1]). A direct role of phosphorylated Tau in neurotoxicity was demonstrated in a study in which degeneration was abolished by blocking all 14 disease-associated phosphorylation sites of Tau in *Drosophila* [2]. A subsequent study showed that coordinated phosphorylation of multiple sites is required to enhance the neurotoxicity [3]. Tau phosphatases, particularly protein phosphatase 2A (PP2A), have been shown to play an important role in Tau-induced toxicity [1]. PP2A is down-regulated while the expression of inhibitors of PP2A, I_1^{PP2A}, and I_2^{PP2A} are up-regulated in AD [4,5]. Inhibition of PP2A by okadaic acid or by expressing I_1^{PP2A}, I_2^{PP2A} and the dominant negative catalytic subunit of PP2A leads to abnormal accumulation of hyperphosphorylated Tau [6,7].

Recent studies have revealed that neurotoxicity of Tau is not entirely dependent on phosphorylation, because oxidative stress has been shown to intensify Tau-induced neurodegeneration without any alteration of Tau phosphorylation [8]. In addition, a mutant Tau resistant to phosphorylation by GSK3β retains substantial toxicity [9]. Interestingly, the mutant Tau species exhibit stronger affinity toward microtubules, implying that microtubule binding is crucial for Tau toxicity [9].

To better uncover the pathogenic pathway and easily identify therapeutic compounds, we have modeled tauopathy in the notum of *Drosophila*. We show that notum is more sensitive and better suited for analyzing Tau-induced toxicity. Additionally, we have demonstrated that the notum-based system is applicable for drug screening, and show the potential of a GSK3β inhibitor as a therapeutic compound.

Materials and methods

Fly stocks. UAS-hTau^{WT}, UAS-hTau^{R406W}, UAS-hTau^{E14}, and UAS-hTau^{AP} were kindly provided by M. Feany [10]. Eq-Gal4 was

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doi:10.1016/j.bbrc.2009.11.089

provided by H. Sun [11]. UAS-Tws was obtained from L.S. Shashidhara [12]. Ppk-Gal4, a dendritic arborizing neuron (da) Gal4 driver, and A101-LacZ, an enhancer reporter line which marks the entire external sensory (ES) organ were provided by C.T. Chien [13]. Gmr-Gal4, $sgg^{M1\text{-}1}$ loss-of-function mutant alleles and dominant negative allele, sgg^{A81T}, tau^{EP3597} mutant, and wdb^{EP3559} alleles were obtained from the Bloomington Stock Center. All fly stocks and genetic crosses were maintained on standard cornmeal-yeast-agar medium at 25 °C unless otherwise mentioned.

Generating of transgenic flies. For generation of UAS-Bβ2 expression constructs, A plasmid containing full length Bβ2 cDNA fused with C-terminal FLAG-GFP was obtained from S. Strack [14]. The DNA fragment containing Bβ2 cDNA with FLAG-GFP tags was digested with BglII and NotI and cloned into the pUAST vector digested with the same restriction enzymes [15]. DNA constructs were confirmed by sequencing before germ-line transformation was undertaken. To generate transgenic flies, a standard germ-line transformation procedure using w^{1118} as the parental line was followed.

Immunoblotting. For protein extraction, 30 dissected nota were homogenized in 100 μL of T-PER tissue extraction buffer (Pierce). Concentrations of extracted protein were quantified using a protein assay kit (Bio-Rad). For each sample, 7.5 μg of total protein was denatured by boiling with Laemmli buffer (2% SDS, 10% glycerol, 0.25 M Tris, 0.01% bromophenol, 5 mM EGTA, 5 mM EDTA, 25 mM DTT, pH 6.8) for 5 min before resolution by SDS–polyacrylamide gel electrophoresis using 12.5% separation gels. Proteins were blotted onto PVDF membranes (Millipore), and the protein blot was blocked in Tris-buffered saline with 0.1% Tween 20 and 5% skim milk. Primary antibodies used in the immunoblotting experiments were as follows: For total Tau protein, mouse anti-Tau5 (1:1000, Biosource) or rabbit anti-Tau (1:5000, DAKO) were used; non-phosphorylated Tau was revealed using anti-Tau1 antibody (1:1000, Chemicon). Antibodies pS199, pS202, pT205, pT231, and pS396 were used to detect different phosphorylated Tau epitopes (Biosource). Appropriate anti-mouse or anti-rabbit HRP-conjugated secondary antibody was applied at a dilution of 1:100,000 (Jackson ImmunoResearch). Signals were detected with ECL kits (Millipore) and captured using an imaging system (Fujifilm LAS-3000).

Alsterpaullone treatment. To administer alsterpaullone to *Drosophila*, a similar protocol was followed as described earlier [16]. Transgenic flies expressing $hTau^{WT}$ were raised at 26 °C on foods containing various concentrations of alsterpaullone (Sigma) dissolved in 100% DMSO.

Bristle quantification and survivorship assay. Nota of one day old adult flies were dissected in PBS with 0.1% Triton X-100. Serial focal plane images of notum bristles were captured by a stereo microscope (Leica MZFLIII) fitted with a digital camera (CoolSnap 5.0, Photometrics). Images were analyzed and bristles were scored using Photoshop software (Adobe). Significance was compared using either one-way ANOVA with supplementary Student–Newman–Kewls (SNK) test (ANOVA/SNK) or Student's *t*-test. For survivorship assay, a group of 10 adult male flies were cultured in a vial. Viable flies were scored and transferred to a new vial in 5 day interval. At least 10 groups of flies were analyzed for each fly line.

Immunohistochemistry and X-gal staining. For visualization of da sensory neurons, larvae were pinned on a Sylgard (Dow Corning) pad, and opened along the ventral midline. The internal organs were removed, and filleted larvae were fixed in 1X PBS containing 3.7% formaldehyde for one hour at room temperature. Fixed larvae were rinsed several times in PBS with 0.3% Triton X-100 and blocked in 5% goat serum. The anti-Tau1 (1: 1000, Chemicon) antibody was used to localize Tau proteins in da neurons, visualized with FITC-conjugated goat anti-mouse secondary antibodies at 1:200 dilution (Jackson ImmunoResearch). Immunostained larvae was captured on a TCS SP2 confocal microscope (Leica Microsystems). For X-gal staining fixed nota were washed briefly with 1X PBS and incubated with X-gal staining solution (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, 3 mM $K_4[Fe(CN)_6]$, 3 mM $K_3[Fe(CN)_6]$, 0.3% Triton X-100, 0.2% X-gal) at 25 °C.

Results

The Drosophila-notum-based system for assaying Tau-induced toxicity

To better evaluate fly models for Tau-associated diseases, we overexpressed wild-type human Tau ($hTau^{WT}$), and various Tau mutants including, $hTau^{R406W}$, $hTau^{V337M}$, phosphorylation-incompetent Tau ($hTau^{AP}$), and pseudophosphorylated Tau ($hTau^{E14}$), in eyes of *Drosophila* under the control of Gmr-Gal4 driver. Compared to the control flies expressing GFP, retinal expression of equivalent levels of Tau variants caused various degrees of rough eye phenotype in flies (Fig. 1A). The most severe rough eye phenotype was found in flies expressing $hTau^{E14}$ or $hTau^{R406W}$ in which the orderly ommatidial architecture was disorganized and photoreceptors were fused with missing mechanosensory bristles (Fig. 1A). Transgenic flies expressing $hTau^{WT}$ showed a moderately rough eye phenotype, and expression of $hTau^{AP}$ and $hTau^{V337M}$ caused mild rough eye phenotypes (Fig. 1A).

The external sensory (ES) organ of *Drosophila* is derived from a sensory organ precursor (SOP). Because of its simplicity in lineage analysis, it has been used extensively for studying pattern formation [17]. To explore whether the ES organ can be used for evaluating Tau toxicity, we overexpressed Tau variants in the notum using Eq-Gal4 driver. Compared to control flies expressing GFP, the expression of Tau variants in the notum resulted in a bristle loss phenotype of varying degrees (Fig. 1A). The loss of notum bristle phenotype was used as a criterion for evaluating the gain-of-toxicity of Tau. A wild-type fly normally bears around 200 evenly-distributed microchaetae on the notum. Overexpression of GFP did not reduce the bristle number (Fig. 1A), but the number of microchaetae fell significantly when wild-type or mutant Tau was overexpressed (Fig. 1A). Quantitative analysis showed that flies expressing $hTau^{R406W}$ exhibited the most severe bristle loss phenotype (Fig. 1B). The expression of $hTau^{WT}$, $hTau^{E14}$ or $hTau^{V337M}$ caused a moderate degree of bristle loss, and flies expressing $hTau^{AP}$ exhibited only a mild bristle loss phenotype. Generally, the overexpression of Tau variants using Gmr-Gal4 or Eq-Gal4 driver presented phenotypes of a similar degree. However, the Gmr-Gal4 driven UAS-$hTau^{E14}$ resulted in severe retinal degeneration, and Eq-Gal4 driving the same transgene induced moderate bristle loss. The disparity of these results may reflect upon the fact that different tissues exhibit different sensitivity to the toxicity of $hTau^{E14}$. As shown above, it is much easier to examine and quantify Tau-induced toxicity using the bristle loss phenotype than the rough eye phenotype. Particularly both ES organ and eye of *Drosophila* contain neurons. We believe that the notum could provide a good alternative for assessing Tau-induced toxicity in flies.

GSK3β and Bβ Subunit of PP2A modulate Tau-induced toxicity in vivo

The role of GSK3β in phosphorylation of Tau and formation of NFT has been well documented [1]. To validate the above findings in our notum-based system, we co-expressed a dominant negative allele of *sgg* (sgg^{A81T}) with $hTau^{WT}$ in the notum of flies. We found that overexpression of sgg^{A81T} mitigated the Tau-induced bristle loss phenotype (Supplementary Fig. S1). In addition, the bristle loss phenotype caused by Tau overproduction was significantly abated in the genetic background of flies heterozygous for the $sgg^{M1\text{-}1}$ mutation (Supplementary Fig. S1). These results indicate that reducing GSK3β activity is beneficial to Tau-induced toxicity. Since our results are in agreement with other studies using photorecep-

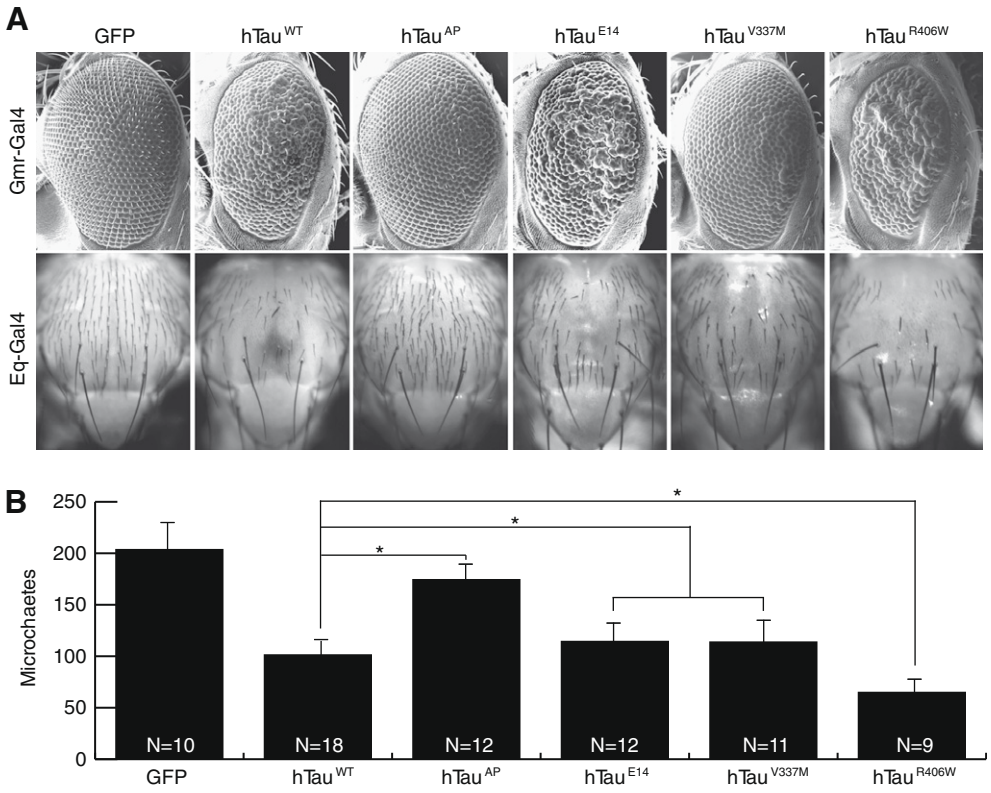

Fig. 1. *Drosophila* models for Tau-induced toxicity. (A) Representative images of *Drosophila* eyes and nota overexpressing GFP, normal human Tau ($hTau^{WT}$), $hTau^{AP}$, $hTau^{E14}$, $hTau^{V337M}$ or $hTau^{R406W}$ driven by Gmr-Gal4 or Eq-Gal4 lines. Control fly expressing GFP shows normal ommatidial architecture and evenly-distributed notum bristles. Overexpression of human Tau variants caused varying degrees of eye degeneration, loss of notum bristle phenotypes. The difference in rough eye phenotype was apparent when Tau variants were driven by Gmr-Gal4 (upper panel). Control fly expressing GFP driven by Eq-Gal4 showed normal bristle pattern. Ectopic expression of Tau variants using Eq-Gal4 driver diminished the notum bristle numbers (lower panel). (B) Quantification of the bristle number of transgenic flies expressing Tau variants assessed by ANOVA followed by Student–Newman–Kewls' (SNK) tests. Asterisks indicate significant differences in bristle counts (*, $P < 0.05$). *N*, number of flies scored from each genotype is indicated in the chart.

tors or wings as disease models [16,18], we conclude that the notum-based system is appropriate for dissecting the underlying mechanisms of Tau mediated toxicity in the context of *Drosophila* genetics.

Since PP2A holoenzyme comprising ABαC or ABβC specifically binds and dephosphorylates Tau specifically *in vitro* [19], it would be interesting to test whether the Bβ regulatory subunit can dephosphorylate Tau and alleviate Tau mediated toxicity *in vivo*. As shown in Fig. 2, overexpression of Tws successfully recovered the Tau-induced bristle loss phenotype (Fig. 2A and B). To further address whether other cell types associated with ES organ were also rescued by expression of Tws, we used the A101 enhancer trap line of *neuralized* to mark sensory cells. Although the sensory cells were distributed evenly in control flies expressing GFP, their number reduced when Tau was overexpressed (Fig. 2A and B). By contrast, bristle and sensory cell numbers increased concurrently when Tws was overexpressed (Fig. 2A and B). A similar rescue effect was observed when human Bβ2 was overexpressed (Fig. 2A), suggesting that function of the Bβ regulatory subunit is evolutionarily conserved. However, the Tau-induced bristle loss phenotype could not be recovered by overexpression of Widerborst (Wdb), a *Drosophila* B′ regulatory subunit homolog, suggesting that PP2A dephosphorylates Tau through specific regulatory subunit (Fig. 2A and B). Immunoblotting experiments were also conducted to determine whether the levels of phosphorylated Tau species were reduced when Tws or Bβ2 were overexpressed. Indeed, overexpression of Tws or Bβ2 reduced the phosphorylation of Tau at pT205, pT231, and pS396 sites, while unphosphorylated Tau was increased dramatically (Fig. 2C). Conversely, levels of phosphorylated Tau species increased significantly when endogenous Tws was silenced by RNAi (Tws-Ri, Fig. 2C). We also overexpressed $hTau^{WT}$ in da neurons of larvae and showed that ectopic Tau formed aggregates and caused dysmorphology of neurites (Fig. 2D). These morphologic defects are likely to have resulted from abnormal Tau aggregation because both pathological features concomitantly existed at the same subcellular locations (Fig. 2D). Additionally, by forcing the expression of Tws we were able to recover the morphology defect (Fig. 2D). These observations clearly demonstrate that Tws/Bβ promotes dephosphorylation of Tau and protects neurons from Tau toxicity.

Reducing endogenous Tau ameliorates Tau-induced toxicity

Overexpression of normal Tau and its isoforms are capable of causing neurodegeneration in transgenic animals [10,18], indicating that elevated Tau itself is a pathogenic factor. It was, therefore, assumed that reducing total levels of Tau would be beneficial. To check this hypothesis, tau^{EP3597}, a protein-null mutant allele of *tau* [20], was crossed with flies overexpressing $hTau^{WT}$. We found that the bristle loss phenotype induced by overexpression of $hTau^{WT}$ was less pronounced in the heterozygous *tau* mutant background (Fig. 3A and B), suggesting that reducing the expression of endogenous Tau is beneficial. Since the levels of phosphorylated Tau species correlated with the pathology, immunoblot analysis was conducted to assess if phosphorylated Tau was inversely proportional to the endogenous Tau level. However, the amount of phosphorylated Tau species was found to be only slightly, but not significantly, lowered in the heterozygous *tau* mutant background (Fig. 3C) indicating that phosphorylated Tau is not the only pathogenic factor and multiple pathogenic pathways are involved in tauopathy.

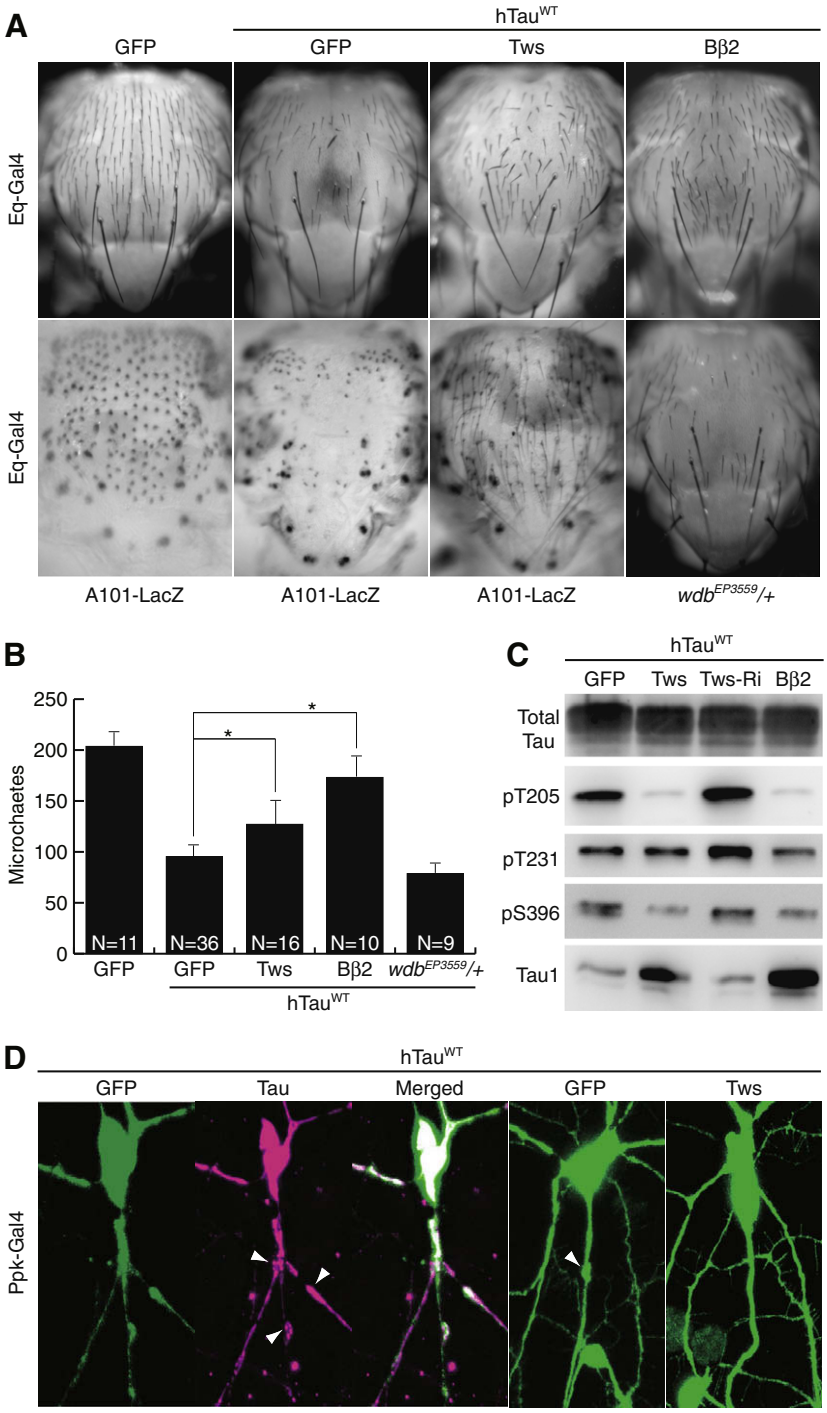

Fig. 2. Bβ regulatory subunit of PP2A rescues Tau toxicity. (A,B) Expression of Tws or Bβ2, but not Wdb restored bristle loss phenotype caused by Tau overexpression. The sensory neurons were revealed by A101 lacZ staining (lower left three panels in A). Loss or recovery of neuron and bristle is correlated. The bristle number did not increase significantly in fly co-expressing Wdb and $hTau^{WT}$ when compared to the control fly expressing GFP and $hTau^{WT}$ (ANOVA/SNK; *, $P < 0.05$). *N*, number of flies scored from each genotype. (C) Immunoblot showed that phosphorylated Tau was reduced when Tws or Bβ2 was overexpressed. By contrast, knocking down endogenous Tws (Tws-Ri) increased phosphorylated Tau species. (D) Overexpression of Tau caused Tau aggregation (magenta) and axonal/dendritic swellings (white arrow head) in da neuron of *Drosophila*. By contrast, elevated Tws activity alleviated the axonal/dendritic atrophy phenotype. (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

Alsterpaullone alleviates Tau-induced toxicity

With the success achieved in identifying genetic components involved in Tau-induced toxicity, we conducted a test to determine whether the notum could serve as a drug screening platform. We have demonstrated that administration of 20–40 μM of alsterpaullone effectively suppressed the loss of bristle phenotype induced by elevated Tau as compared to control flies raised on food with or without DMSO (Fig. 4A). The bristle number increased proportionally to the concentration of the inhibitor, suggesting that the beneficial effect of alsterpaullone is concentration-dependent (Fig. 4B). Since neuronal overexpression of $hTau^{R406W}$ exhibits stronger toxicity in fly, to further evaluate the effectiveness of alsterpaullone we oral administration of the compound to trans-

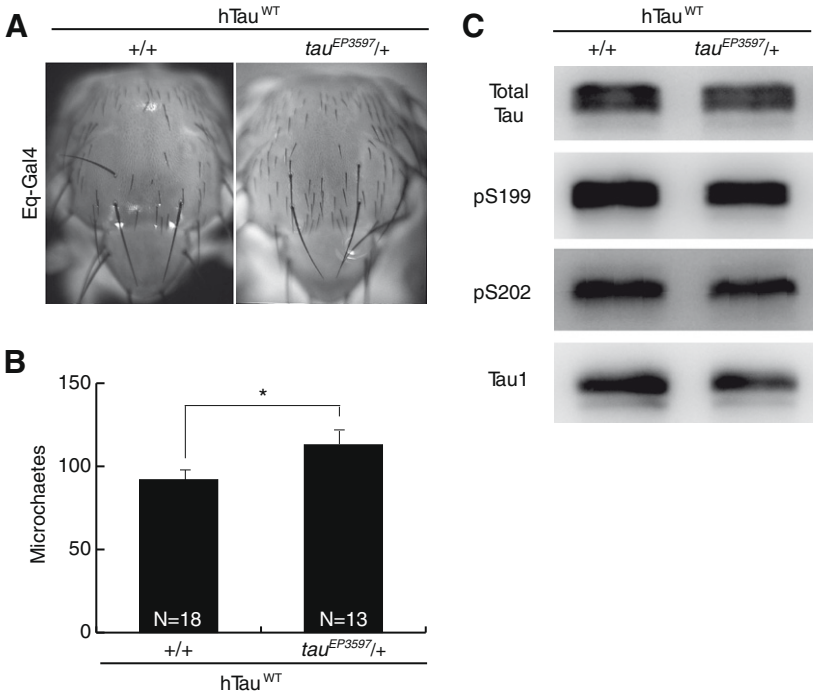

Fig. 3. Reducing endogenous Tau suppresses Tau-induced toxicity. Genetic removal of one copy of *tau* abated bristle loss phenotype. (A) Light images of adult notum showing that bristle loss phenotype caused by Tau expression was abated under reduced *tau* background were supported by quantitative analysis (B) of the number of microchaetae (Student's *t*-test; $^{*}P < 0.05$; *N*, number of flies scored from each genotype). (C). Immunoblotting showed that phosphorylated Tau was not significantly reduced when endogenous *tau* dosage was reduced.

genic fly overexpressing $hTau^{R406W}$ driven by pan-neuronal Elav-Gal4. As shown in Fig. 4C, 10 μM significantly extended the lifespan of fly model (Fig. 4C). Although a higher concentration of alsterpaullone (20 μM) showed adverse effects (Fig. 4C), alsterpaullone can be considered as a lead drug for treating tau-induced toxicity. Taking together, we concluded that the notum-based system can provide a fast screening platform for discovering therapeutic compounds for Tau related diseases.

Discussion

The fly *Drosophila* has been demonstrated to be one of the most valuable animal models for the study of neurodegeneration. To model Tau-induced toxicity in *Drosophila*, normal and mutant forms of the human Tau have been expressed in the photoreceptors or neurons [10,18]. Although tauopathy models based on degeneration of photoreceptor provide a convenient way of evaluating pathological presentation, it is somewhat subjective to judge the degree of degeneration based on a single morphological criterion, because retinal overexpression of pathogenic proteins results in various combinations of morphological defects including depigmentation, disorganization and fusion of ommatidia, reduced size of eye and thickness of retina, as well as loss of bristle and photoreceptor neurons. In some cases, unrelated pathology, such as lethality, can be observed. In addition, certain anatomic and imaging technologies, including SEM, TEM or immuncytochemistry staining are required to assess the detailed pathological phenotypic changes, thereby contributing to the difficulty in analyzing the gain-of-toxicity function of the disease-causing proteins. In this study we have explored the possibility of using the fly notum to model Tau mediated toxicity in *Drosophila*. Targeted expression of human Tau protein in the notum resulted in loss of the entire ES organs of adult flies (Figs. 1A and 2A). Nevertheless, the notum itself was not altered when considering its overall shape and size upon Tau overexpression (Fig. 1A). Compared to other *Drosophila* tauopathy models, the bristle loss phenotype in the notum-based model also has a higher penetrance, and importantly, the severity of phenotype is highly correlated with the toxicity of Tau (Fig. 1). It is, therefore, we established that the notum is a better system for modeling Tau-induced toxicity in flies.

Since the formation of NTF is one of the key pathologic features of tauopathies, we have attempted to detect such pathology in the notum of *Drosophila* overexpressing Tau. Although punctuate staining was noted in the notum of fly expressing $hTau^{WT}$, we were unable to detect any NFT-like structure (Supplementary Fig. S2 and data not shown). Our results corroborate with previous studies in which Tau-immunoreactive filaments were not identified in the brain of transgenic flies expressing Tau variants [10,21]. It was suggested that Tau-induced degeneration is independent of the presence of NFT [10]. Nevertheless, we found that overexpression of $hTau^{WT}$ lead to aggregation and neuronal dystrophy in da neurons (Fig. 2D). Our results further strengthen the notion that different cell types exhibit different responses to Tau toxicity. Thus, it is advisable to analyze Tau toxicity using different model systems.

Down-regulation of the Bβ regulatory subunit of PP2A has been found to be correlated with the incidence of AD [22]. In the present study, we have found that ectopic Tws or Bβ2 activity can rescue Tau mediated toxicity. Our results were consistent with previous findings in which PP2A comprised of ABβC bound and dephosphorylated Tau specifically *in vitro* [19]. Ectopic expression of the B' subunit homolog, Wdb, was unable to recover the notum bristle loss phenotype in flies (Fig. 2A and 2B). Nevertheless, a previous study showed that down-regulation of PP2A by overexpressing a dominant negative Wdb construct resulted in increased levels of phosphorylated Tau and neurotoxicity [2]. Notably, gain of Wdb function also enhanced Tau-induced toxicity in a modifier screen [23]. Since PP2A comprised of AB′C cannot dephosphorylate Tau effectively under *in vitro* condition [19], and Wdb itself is likely to be involved in eye development of *Drosophila* because retinal overexpression of Wdb alone resulted in rough eye phenotype

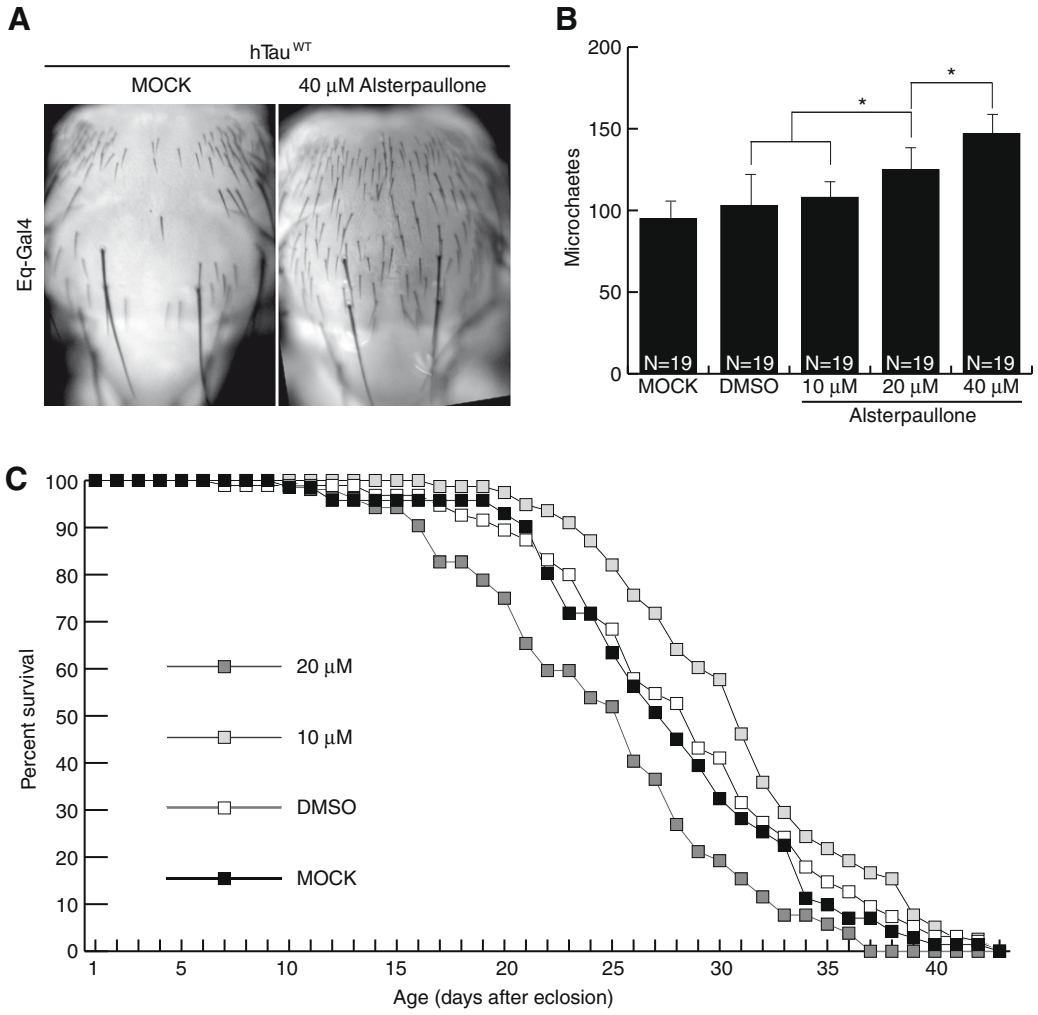

Fig. 4. Alsterpaullone alleviates Tau-induced toxicity. (A) The control flies (MOCK) showed bristle loss phenotype due to overexpression of $hTau^{WT}$ (left panel). Administration of alsterpaullone effectively rescued Tau-induced bristle loss phenotype (right panel). (B) The beneficial effect of alsterpaullone was dose-dependent (ANOVA/SNK; $^{*}P < 0.05$; $N = 19$ for each genotype). (C) Neuronal overexpression of $hTau^{R406W}$ by Elav-Gal4 driver caused premature death. A low concentration of alsterpaullone (10 μM) extended the lifespan of transgenic flies, while higher concentrations of alsterpaullone (20 μM) shortened lifespan.

(Supplementary Fig. S3), we believe that Tau is unlikely to be a direct target of Wdb.

Tauopathy animal models can be generated by overexpressing normal Tau or its splicing variants, suggesting that elevated Tau can cause tauopathy. Here, we demonstrate that reducing endogenous Tau levels partially recovered the bristle loss phenotype (Fig. 3A). Nevertheless, phosphorylated Tau species were not reduced significantly (Fig. 3C), suggesting that down-regulation of endogenous Tau of *Drosophila* does not alter the phosphorylation status of exogenous Tau, and phosphorylated Tau species might not be the only pathogenic factor of tauopathies.

A convenient and quantifiable assay system for Tau mediated neurodegenerations will greatly assist in evaluating pharmacologic treatments for the diseases. In the current study, we chose a GSK3β inhibitor to test our model. This was based on the fact that GSK3β is a major Tau kinase that promotes Tau toxicity, and GSK3β inhibitors have been shown to be very effective in suppressing Tau-induced toxicity [16,24]. We have shown in this study that alsterpaullone rescues the Tau-induced bristle loss phenotype in a dosage-dependent manner (Fig. 4B). Eventhough we have only demonstrated the usefulness of our system using a single compound, the simplicity and sensitivity of the system leaves us confident that it is possible to use this system for large-scale screening of drug.

Acknowledgments

We thank Mel Feany, Henry Sun, L.S. Shashidhara, Cheng-Ting Chien and Bloomington Stock Center for fly stocks; Jyh Lyh Juang, Stefan Strack and Developmental Studies Hybridoma Bank for cDNA clones and reagents; and Wei-Jun Chien for technical assistance. We thank Ching-Jin Chang, Pang-Hsien Tu, Harry Wilson, Henry Sun, Margaret Ho, and Yijuang Chern for their valuable comments. This work was supported by grants to M.T.S. from the National Science Council (95-2311-B-003-006-MY2; 96-2311-B-003-005) and National Taiwan Normal University (97C05) of the Republic of China.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.089.

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