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Note

Interaction Between Eye Pigment Genes and Tau-Induced Neurodegeneration in *Drosophila melanogaster*

Surendra S. Ambegaokar* and George R. Jackson†,[1]

**Neuroscience Interdepartmental Ph.D. Program, Brain Research Institute, Center for Neurobehavioral Genetics, Semel Institute for Neuroscience and Human Behavior, and Department of Neurology, David Geffen School of Medicine, University of California, Los Angeles, California 90095 and* †*Departments of Neurology, Neuroscience & Cell Biology, and Biochemistry and Molecular Biology, and Mitchell Center for Neurodegenerative Diseases, University of Texas Medical Branch, Galveston, Texas 77555*

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ABSTRACT

Null mutations in the genes *white* and *brown*, but not *scarlet*, enhance a rough eye phenotype in a *Drosophila melanogaster* model of tauopathy; however, adding *rosy* mutations suppresses these effects. Interaction with nucleotide-derived pigments or increased lysosomal dysregulation are potential mechanisms. Finally, tau toxicity correlates with increased GSK-3β activity, but not with tau phosphorylation at Ser202/Thr205.

IN transgenic models of tauopathy in *Drosophila melanogaster*, transgenes are often introduced into a "white" genetic background, which is a homozygous null allele of the *white* gene classically known for its role in eye pigmentation. Here, we demonstrate that *white*, as well as *brown* and *rosy*, two other pigment-related genes, dose dependently affect the tau-induced eye phenotype, tau phosphorylation, and GSK-3β activity. The effects of these pigment-related genes are not light dependent, suggesting involvement of other cellular mechanisms, such as increased lysosomal dysregulation or interaction of tau with pigment or pigment precursor molecules, *e.g.*, drosopterins, which might induce tau seeding and aggregation. Additionally, tau toxicity correlates with increased GSK-3β/Shaggy activity, but not with tau phosphorylation at Ser202/Thr205, suggesting a role of GSK-3β activity in regulating tau toxicity independent from its ability to phosphorylate tau at S202/T205 and also implying the ability of *white*, *brown*, and *rosy* to regulate GSK-3β activity.

The red eye of *D. melanogaster* is rendered white by homozygous mutation of the *white* (*w*) gene. *P* elements, naturally occurring transposable elements in Drosophila, can be modified to carry transgenes (Rubin and Spradling 1983) and used for mutagenesis by inserting into genomic regions (Cooley *et al.* 1998a,b). The *white* mutant background is commonly used for insertion of transgenes that carry a cDNA sequence of the wild-type *white* gene, *mini-white* (w^{+mC}), which serves as a positive marker of transgene incorporation (Klemenz *et al.* 1987). One copy of w^{+mC} is sufficient to induce red eye development in a *white* homozygous mutant, although the degree of pigmentation may vary depending on insertion position. We created a model of tau-induced toxicity in Drosophila by expressing full-length, wild-type human tau in the eye of the fly, directly fusing the tau cDNA to the eye-specific *glass* (*gl*) promoter ("*gl*-tau" fly), which yields a rough eye phenotype (Jackson *et al.* 2002). This transgene is in a w^{1118} homozygous background and carries one copy of w^{+mC}. While conducting a genetic screen for modifiers of tau toxicity using *P*-element insertion mutants, we observed that the rough eye phenotype was affected by the *P* elements themselves and hypothesized that this effect was due to the additional w^{+mC} carried on the *P* element. To test this hypothesis, we crossed our *gl*-tau fly to a w^+ strain (Canton-S) and compared the *gl*-tau phenotype in a w^{1118} homozygous background to that obtained in a w^+/w^{1118} heterozygous background. The w^{1118} homozygotes showed a significant reduction in eye size and increased ommatidial disorganization as compared to w^+/w^{1118} heterozygous flies (Figure 1, B and C). Furthermore, w^{1118} homozygous eyes often had necrotic plaques, which were never observed in w^+/w^{1118} heterozygotes. To more accurately describe levels of degeneration, we utilized the Nikon AZ100M microscope NIS-Elements AR 3.0 software (Nikon Instruments, Melville, NY), which features an "extended depth of focus" (EDF) algorithm that allows for three-dimensional reconstruction, as demonstrated in Figure 2, A–C. This imaging allows

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[1]*Corresponding author:* The University of Texas Medical Branch, 301 University Blvd., MRB 10.138, Galveston, Texas 77555-1045.
E-mail: grjackso@utmb.edu

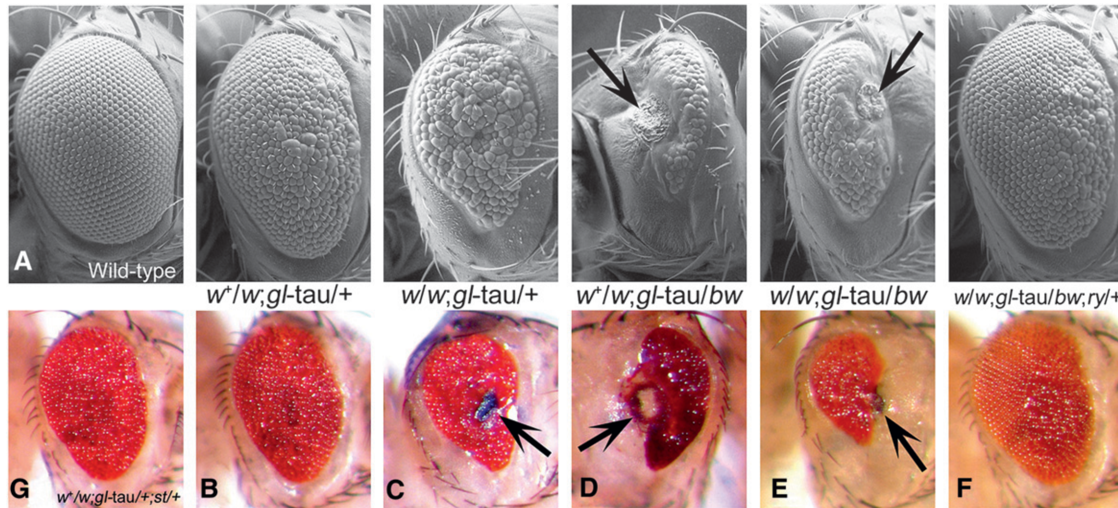

Figure 1.—Scanning electron micrographs (SEMs) and color light micrographs demonstrating null alleles of *white* (*w*) and *brown* (*bw*) enhance tau-induced toxicity. Arrows: necrotic patches. (A) Wild-type (Canton-S). (B) *white* heterozygote: w^{+}/w^{1118}; *gl*-tau/+. (C) *white* homozygote: w^{1118}; *gl*-tau/+. (D) *brown* allele: w^{+}/w^{1118}; bw^{1}/*gl*-tau. (E) *white* homozygote + *brown*: w^{1118}; bw^{1}/*gl*-tau. (F) Null allele of *rosy* revert *white* and *brown* enhanced toxicity: w^{1118}; bw^{1}/*gl*-tau; ry^{506}/+. (G) Null mutations in *scarlet* (*st*) do not affect tau-induced toxicity: w^{+}/w^{1118}; *gl*-tau/+; st^{1}/+. Flies were anesthetized with carbon dioxide for light microscopy images, taken with a digital-camera equipped Zeiss dissecting microscope. Flies were dehydrated in hexamethyldisilazane prior to mounting for SEM, as previously described in Jackson *et al.* (2002). SEM images were taken on a Hitachi S-2460N scanning electron microscope. Stocks and crosses were maintained on a standard yeast-molasses-cornmeal medium at 23° or 25°.

for estimated eye-volume calculations and can be used in our tauopathy model as a metric for degeneration. Figure 2D plots the mean total eye volumes of all the genotypes used in this study, with the actual volume means and *P* values for each pairwise genotypic comparison listed in Table 1.

To rule out background effects in the w^{1118} line, the *gl*-tau fly was crossed to another *white* allele line, w^{2202}, to create *white trans*-homozygotes (w^{1118}/w^{2202}); tau enhancing effects comparable to those seen in w^{1118} homozygous flies were observed (Figure 2D; Table 1). This effect is dose dependent, as increasing copies of w^{+mC} further suppressed the *white* mutation-induced toxicity. The *white* gene encodes an ATP binding cassette cotransporter (ABC transporter) that is expressed in many tissues in Drosophila (O'Hare *et al.* 1984; Mount 1987; http://flyatlas.org). In the eye, White is coupled to either Brown or Scarlet—both also ABC transporters—to transport one of two types of pigment molecules into pigment granules. White and Brown transport guanine-derived drosopterin precursors, while White and Scarlet transport tryptophan-derived xanthommatin precursors (Dreesen *et al.* 1988; Tearle *et al.* 1989; Mackenzie *et al.* 2000). We tested whether mutations in *brown* (*bw*) or *scarlet* (*st*) exerted effects similar to those of *white* mutations. Although no significant effect of st^{1} was found (Figure 1G), the bw^{1} allele greatly enhanced tau-induced toxicity, producing severe eye reduction and large necrotic patches (Figure 1D), demonstrating specificity of the enhanced toxicity to *brown* and *white*, but not *scarlet*. One copy of bw^{1} was sufficient to induce this phenotype in a w^{+}/w^{1118} background. A similar degree of toxicity was observed with a single bw^{1} null allele in a w^{1118} homozygous background ("*white* + *brown*") (Figure 1E; Figure 2D). Although mutant homozygous *white* pigment phenotypes are epistatic to mutant *brown* and *scarlet* pigment phenotypes, the lack of an epistatic effect of *white* with *brown* with tau, in addition to the lack of a phenotype with a *st* mutation, indicates a specific role of drosopterins or drosopterin precursors transported by Brown in enhancing tau toxicity and not general effects by all pigment precursors or pigment granules. To rule out potential background effects from the bw^{1} line, other *brown* alleles were tested and also showed enhanced toxicity, albeit less severe than those observed with bw^{1} (bw^{19} and bw^{16}; Figure 2D and Table 1). From these observations, it can be concluded that loss-of-function mutations in *white* and *brown* enhance tau-induced toxicity.

We hypothesized that reductions in White and Brown impair transport of drosopterins or their precursors into pigment granules, causing their cytosolic accumulation. The gene *rosy* (*ry*) encodes xanthine dehydrogenase (XDH), which is found in pigment granules that contain Brown but not those that contain only Scarlet (Reaume *et al.* 1991). Although the role of XDH in pigmentation is complex, it is clear that *rosy* mutations are associated with decreased drosopterin levels, suggesting that *rosy* is involved in producing pterins transported by Brown and White. One copy of mutant *rosy* (ry^{506}) was sufficient to revert the enhanced toxicity of *white* homozygotes and *white* + *brown* flies (Figure 1F), although *white* + *brown* + *rosy* flies were not completely rescued to the levels of *white* + *rosy* alone (Figure 2D), emphasizing the strength

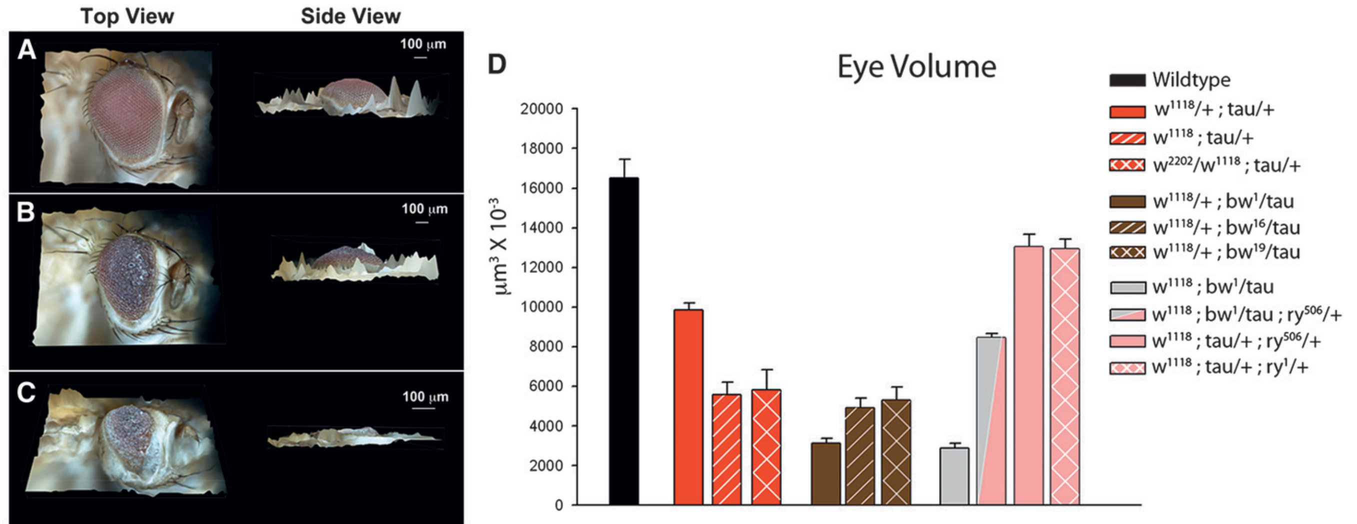

Figure 2.—Three-dimensional reconstructions of eye volumes (A–C) were obtained using a Nikon AZ100M light microscope and Nikon DS-Fi1 digital camera with EDF algorithm with Nikon NIS-Elements AR 3.0 software on Z-stack planar images. Stacks were created by 10-μm intervals between planes; area and volume per plane were obtained by software analysis determined by region of interest boundaries. Top-down and side views are shown to demonstrate 3D reconstructions and observable differences in eye volume due to tau toxicity. (A) Wild-type eye. (B) *white* heterozygote eye: w^+/w^{1118}; *gl*-tau/+. (C) *white* homozygote: w^{1118}; *gl*-tau/+. (D) Mean total eye-volume plot of genotypes described in this study. *P* values for each pairwise comparison are found in Table 1. Graph was created with SigmaPlot 9.0 (Systat, San Jose, CA).

of degeneration that *brown* induces. To rule out potential background effects from the ry^{506} line, a different *rosy* allele, ry^1, was tested; it suppressed the enhanced toxicity of *white* homozygotes flies nearly identically to the ry^{506} allele (Figure 2D and Table 1).

The use of the *glass* promoter induces expression in many cell types of the eye (Ellis *et al.* 1993), including photoreceptors, cone cells, and pigment cells, in which the majority of pigment granules are found. Pigment granules function to optically isolate each ommatidium and reduce excess exposure to light (Kirschfeld and Franceschini 1969; Franceschini and Kirschfeld 1976). To test whether the effects on tau toxicity due to mutations in *white* and *brown* were due to a reduced facility for light absorption, or were photoreceptor-activity dependent, flies were reared in 24 hr darkness from embryo until 2–3 days post-eclosion (dark reared) and compared to flies grown in a 12-hr light/12-hr dark cycle but kept in otherwise identical environmental conditions (light reared). As seen in Figure 3A, the effects of *white* and *brown* were identical in dark reared and light reared *gl*-tau flies; eye-volume calculations between groups demonstrate no difference between dark- and light-reared flies; moreover the enhanced toxic effects of *white* and *brown* are present even in the absence of light (Figure 3B). We conclude that tau toxicity itself, and the effect of *white* and *brown* mutations on tau, are light independent, suggesting that cellular functions of *white* and *brown* apart from photoreceptor isolation and protection modify tau toxicity (discussed further below). As the majority of pigment granules are in pigment cells, it is reasonable to conclude that the synergistic toxic tau effects are more abundant in pigment cells. However, given that a small number of pigment granules are also found in photoreceptors (Kirschfeld 1979; Hofstee and Stavenga 1996), as well as the light independence of the phenotypes, we cannot rule out that the tau-*white*–*brown*-enhanced degeneration is present in photoreceptors and other cell types as well, nor that nonautonomous cell-induced degeneration is also occurring. Indeed, mRNA for *white*, *rosy*, and *brown* is enriched in Malpighian tubules (Chintapalli *et al.* 2007), and the *white* gene product, at least, is involved in the transport of important regulatory molecules (Evans *et al.* 2008). Thus, it is entirely possible that "eye-color" genes affect retinal degeneration indirectly via their influence on synthesis and transport of molecules that circulate in hemolymph and are taken up by tau-producing cells.

Tauopathies are neurodegenerative diseases characterized in part by hyperphosphorylated intracellular aggregates of the microtubule-associated protein tau. Tau phosphorylation at S202/T205, as detected by the AT8 antibody (Biernat *et al.* 1992), is a common feature in tauopathies and accumulates in fairly late neurofibrillary tangle development (Ikura *et al.* 1998; Wada *et al.* 1998; Augustinack *et al.* 2002; Ferrer *et al.* 2002; Wray *et al.* 2008; Han *et al.* 2009). Thus, increased AT8 signal is predicted to correlate with enhanced toxicity found in *white* and *white* + *brown* backgrounds; however, AT8 immunoreactivity was reduced in w^{1118} homozygous flies as compared to w^+/w^{1118} heterozygotes (Figure 4A). Furthermore, AT8 signal was barely detectable in *white* + *brown* flies—a genotype with the strongest level of degeneration; however, one copy of ry^{506}, which reduced cellular degeneration, again surprisingly increased AT8 signal in *white* + *brown* flies to levels comparable to those

TABLE 1

Eye-volume statistics

A. Volume measurements ($\mu m^3 \times 10^{-3}$)

	Wild type	*w*/+	w^{1118}	w^{2202}	bw^1	bw^{16}	bw^{19}	*w,bw*	*w,bw,ry*	w,ry^{506}	w,ry^1
Mean	16514.7	9860.0	5595.6	5815.7	3133.6	4903.0	5302.5	2881.0	8446.9	13048.7	12936.8
n	5	8	8	5	7	8	9	7	8	9	8

B. Pairwise comparisons *P*-values: ANOVA with Holm–Sidak test for significance ($P < 0.01$)

	Wild type	*w*/+	w^{1118}	w^{2202}	bw^1	bw^{16}	bw^{19}	*w,bw*	*w,bw,ry*	w,ry^{506}	w,ry^1
Wild type	–	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
w/+		–	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.063	<0.001	<0.001
w^{1118}			–	0.797	0.002	0.358	0.689	<0.001	<0.001	<0.001	<0.001
w^{2202}				–	0.003	0.289	0.541	0.001	0.003	<0.001	<0.001
bw^1					–	0.026	0.005	0.753	<0.001	<0.001	<0.001
bw^{16}						–	0.585	0.011	<0.001	<0.001	<0.001
bw^{19}							–	0.002	<0.001	<0.001	<0.001
w,bw								–	<0.001	<0.001	<0.001
w,bw,ry									–	<0.001	<0.001
w,ry^{506}										–	0.878
w,ry^1											–

Statistical analysis performed with SigmaPlot 9.0. All listed genotypes other than "Wild type" contain 1 copy of *gl*-tau transgene.

observed in w^+/w^{1118} flies (Figure 4A). Total tau levels as detected by the T46 antibody (Kosik *et al.* 1988; Bramblett *et al.* 1993) were similar between genotypes.

This lack of correlation of tau phosphorylation with phenotype prompted us to investigate the activation state of glycogen synthase kinase-3β beta (GSK-3β), which is known to target the Ser202/Thr205 site recognized by AT8 (Mandelkow *et al.* 1992). GSK-3β is a constitutively active kinase that is inactivated when phosphorylated at serine-9 (Sutherland *et al.* 1993). Immunoblots using an antibody specific to phospho-GSK-3β^{Ser9} revealed strong decreases in inhibitory GSK-3β phosphorylation in *white* and *white* + *brown* flies, indicating increased activity. However, one copy of ry^{506}

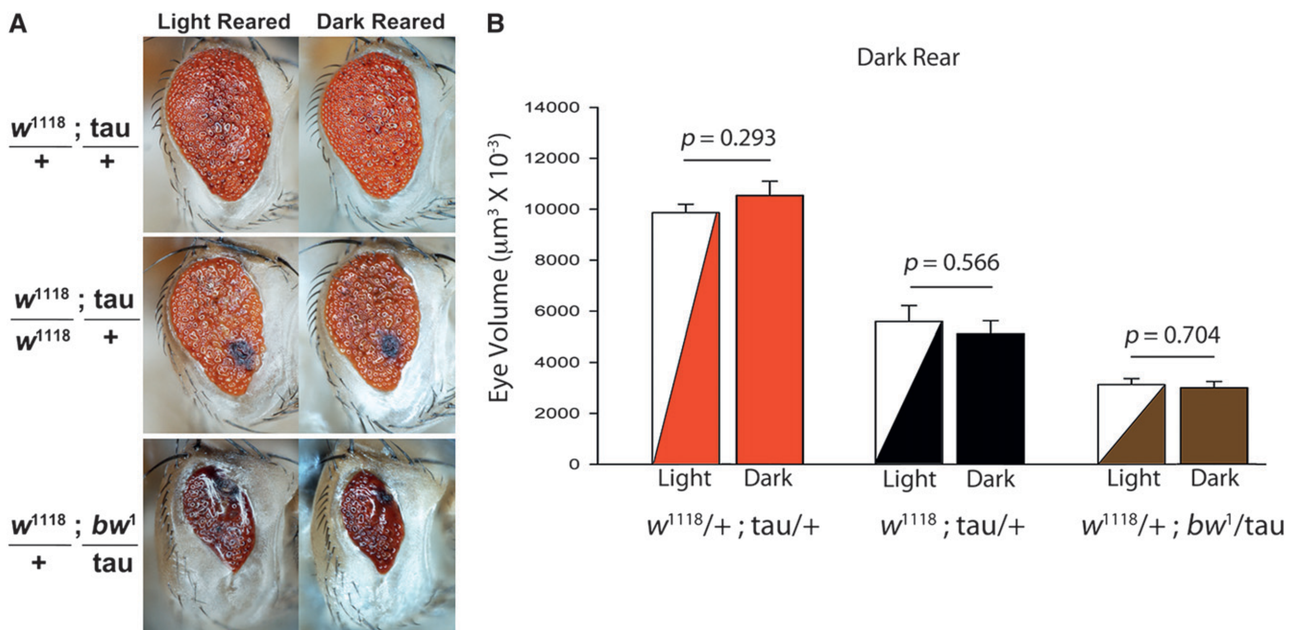

Figure 3.—Tau toxicity modulation by *white* and *brown* is light independent. (A) Light micrographs of *gl*-tau flies reared in 24 hr darkness from embryo to 2–3 days posteclosion (dark reared) as compared to *gl*-tau flies reared in 12-hr light/12-hr dark cycle under identical environmental conditions (22°, ambient humidity). No phenotypic difference due to light was observed in *white* heterozygotes (top row, w^+/w^{1118}; *gl*-tau/+), *white* homozygotes (middle row, w^{1118}; *gl*-tau/+), or *brown* genotypes (bottom row, w^+/w^{1118}; bw^1/*gl*-tau). Images taken with Nikon AZ100M microscope equipped with Nikon DS-Fi1 digital camera. (B) Eye-volume measurements show no statistical difference within genotypes between light *vs.* dark reared flies. *P* values were determined by *t*-test (SigmaStat 11.0, Systat, San Jose, CA) and graphs were created using SigmaPlot 9.0.

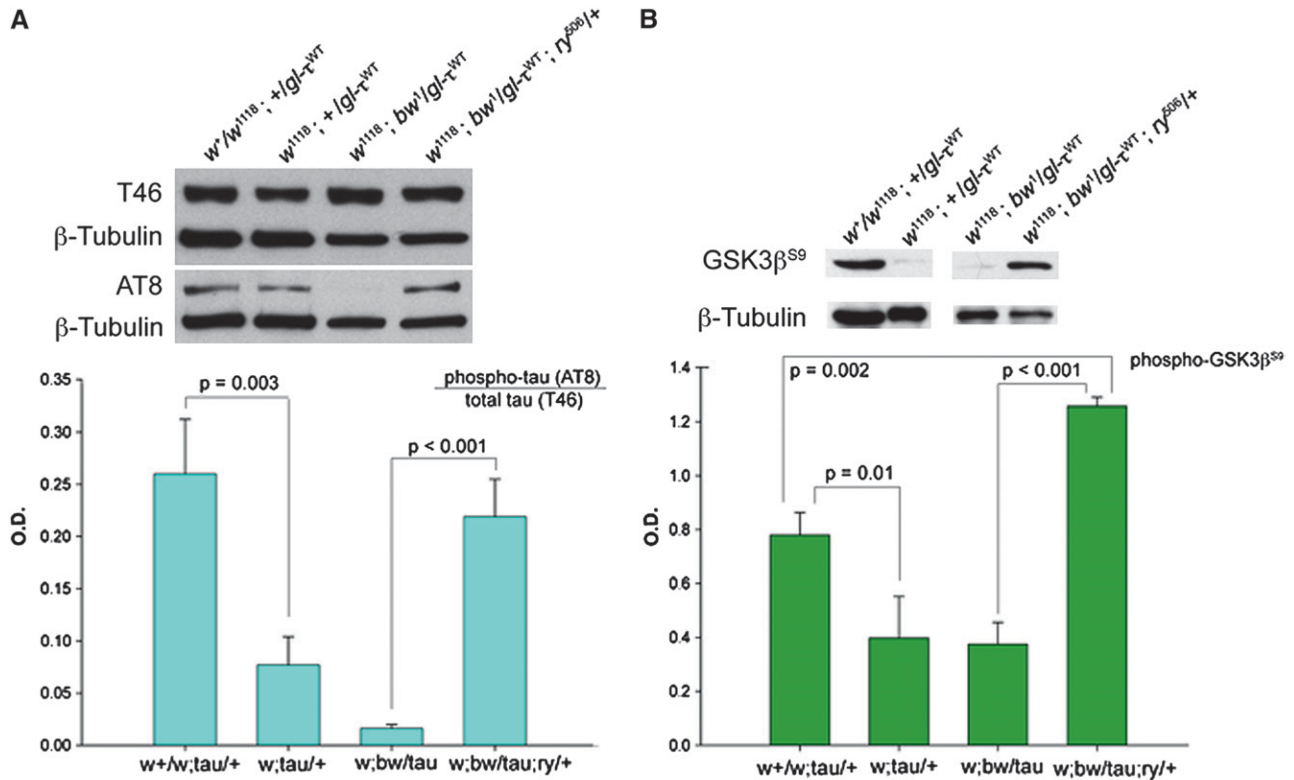

Figure 4.—*rosy* restores tau phosphorylation and decreases inhibitory GSK-3β phosphorylation. (A) *white* homozygote and *white* + *brown* show reduced S202/T205 phosphorylation (AT8 antibody, Pierce/Thermo Scientific, Rockford, IL), which is restored with $rosy^{506}$ allele. Total tau levels are similar (T46 antibody, Invitrogen, Carlsbad, CA). (B) Phosphorylation of GSK-3β at Ser9, which inactivates GSK3β, is reduced in *white* homozygotes and *white* + *brown*, indicating increased GSK-3β activity; Ser9 phosphorylation is restored by a mutation in *rosy* (phospho-GSK3β-Ser9 antibody, GeneTex, Irvine, CA). Protein was extracted from fly heads and processed in TBS buffer with phosphatase and protease inhibitors (Roche Diagnostics, Manheim, Germany) and run on 10–20% SDS–PAGE gels (Bio-Rad, San Diego, CA). β-Tubulin is shown as loading control (Accurate Chemical, Westbury, NY). *P* values were determined by *t*-test (SigmaStat 11.0) and graphs were created with SigmaPlot 9.0 (Systat, San Jose, CA).

greatly increased inhibitory phosphorylation of GSK-3β in a *white* + *brown* background ($P < 0.001$; Figure 4B).

We derive four conclusions from these data:

1. S202/T205 phosphorylation does not correlate well with severity of tau phenotypes in our model. Recent studies using tau constructs resistant to phosphorylation also demonstrated uncoupling of tau phosphorylation at S202/T205 and toxicity (Steinhilb *et al.* 2007; Chatterjee *et al.* 2009), suggesting that other mechanisms, such as increased microtubule binding affinity by tau (Chatterjee *et al.* 2009), or alternatively, tau oligomerization (Kayed and Jackson 2009), may have more direct toxic effects.
2. GSK-3β activity does not correlate with *in vivo* phosphorylation of S202/T205, suggesting that other kinases may outcompete GSK-3β *in vivo* for tau phosphorylation at the S202/T205 sites. Some putative competing kinases are cyclin-dependent kinase 5 (Paudel *et al.* 1993) or extracellular regulated kinase (Drewes *et al.* 1992); each has been shown to also target the S202/T205 sites.
3. GSK-3β activation state correlates well with tau toxicity, with lower activity state correlated with reduced toxicity. This suggests that GSK-3β activity modulates tau-induced toxicity through mechanisms independent of direct S202/T205 phosphorylation. GSK-3β has several downstream targets and is a regulator in many pathways, including Wnt, PI3K, and hedgehog signaling (Liang and Slingerland 2003; Cadigan and Liu 2006; Wang *et al.* 2007). One such target is the cotranscription factor, Armadillo, which we have previously shown to modulate tau toxicity (Jackson *et al.* 2002). GSK-3β may also modulate tau-induced toxicity by regulating the activity of the kinase *partitioning defective 1* (*par-1*) (Timm *et al.* 2008). PAR-1, also known as MARK (Microtubule-Associated Protein/Microtubule Affinity Regulating Kinase), is another known tau kinase (Drewes *et al.* 1995) shown to modulate tau-induced toxicity; however, reports differ as to whether PAR-1 activity enhances (Nishimura *et al.* 2004; Chatterjee *et al.* 2009) or suppresses (Shulman and Feany 2003; Chen *et al.* 2007; Thies and Mandelkow 2007) tau-induced toxicity.
4. Mutations in *white*, *brown*, and *rosy* can affect GSK-3β activity, although more work is needed to understand the mechanisms behind this regulation.

Human homologs of *rosy*/XDH and *white* (*ABCG1*) have been cloned and mapped, and both are expressed in several tissues including the brain (Ichida *et al.* 1993; Xu *et al.* 1994; Chen *et al.* 1996; Croop *et al.* 1997; Saksela *et al.* 1998). Mutations in *ABCG1* are associated with mood and panic disorders (Nakamura *et al.* 1999), and mutations in *white* and *brown* have a range of neurobehavioral effects in flies, including reduced sensitivity to anesthesia, learning defects, and abnormal courtship behavior (Zhang and Odenwald 1995; Campbell and Nash 2001; Diegelmann *et al.* 2006). Our data fit a model in which an enzymatic product of XDH is transported by White and Brown into granules and in which interaction of this product with tau is detrimental to the cell. Drosopterins are derived from the nucleotide guanosine-5′-triphosphate (GTP), and it has been demonstrated that mammalian tau can interact directly with nucleic acids (Schröder *et al.* 1984; Wang *et al.* 2006; Sjöberg *et al.* 2006); thus it is conceivable that nucleotides and derivatives such as drosopterins directly interact with tau and induce aggregation. This association could also sterically inhibit the interaction of kinases with tau, causing a reduction of AT8 signal.

An alternative model is one of White reduction and tau-overexpression synergism in lysosomal dysregulation. In Drosophila, *white* mutants have abnormally large pigment granules. Granules with improper pigment balance due to *white*, *brown*, or *scarlet* mutations become autolysosomes (Shoup 1966; Stark and Sapp 1988). Lysosomal dysregulation is a characteristic feature of Niemann–Pick disease type C and Sanfillipo syndrome type B, both tauopathies (Blanchette-Mackie *et al.* 1988; Sokol *et al.* 1988; Suzuki *et al.* 1995; Ohmi *et al.* 2009). Additionally, Dermaut *et al.* (2005) showed that abnormal loss-of-function mutations in *benchwarmer*, which are associated with enlarged lysosomes, also dose dependently enhance tau toxicity. Lysosomal degradation of tau may be an important method of tau clearance; thus, dysfunctional lysosomes may exacerbate tau toxicity. It has been suggested that XDH is required for the formation of the pigment granules that contain Brown and White (Reaume *et al.* 1991); thus, *rosy* null mutations may rescue *white* and *brown* enhanced tau-induced toxicity by preventing the formation of granules that would otherwise become abnormal and autophagic. It may be that the strong reduction in Ser202/Thr205 tau phosphorylation seen with *white* and *brown* mutations is due to tau being sequestered into large but dysfunctional lysosomal/autophagosomal bodies where it is protected from kinase activity.

The results presented here identify novel genetic modifiers of tau-induced toxicity that have with human homologs; such modifiers may function to increase seeding for tau aggregation, augment lysosomal dysregulation, or both. In addition, these data also suggest a novel connection between *white*, *brown*, and *rosy* and GSK-3β/*shaggy* activity. Finally, given the common use of these eye-color mutations as genetic backgrounds for identification of *P*-element transformants, these results also have important implications for interpreting genetic disease models in Drosophila.

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