Genetic Modifiers of Tauopathy in Drosophila

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ABSTRACT

In Alzheimer's disease and related disorders, the microtubule-associated protein Tau is abnormally hyperphosphorylated and aggregated into neurofibrillary tangles. Mutations in the *tau* gene cause familial frontotemporal dementia. To investigate the molecular mechanisms responsible for Tau-induced neurodegeneration, we conducted a genetic modifier screen in a Drosophila model of tauopathy. Kinases and phosphatases comprised the major class of modifiers recovered, and several candidate Tau kinases were similarly shown to enhance Tau toxicity *in vivo*. Despite some clinical and pathological similarities among neurodegenerative disorders, a direct comparison of modifiers between different Drosophila disease models revealed that the genetic pathways controlling Tau and polyglutamine toxicity are largely distinct. Our results demonstrate that kinases and phosphatases control Tau-induced neurodegeneration and have important implications for the development of therapies in Alzheimer's disease and related disorders.

ALZHEIMER'S disease is the most common neurodegenerative disorder and causes progressive memory loss eventually culminating in severe cognitive dysfunction and death. Dementia is accompanied pathologically by neuronal loss and the diagnostic hallmarks of Alzheimer's disease: amyloid plaques and neurofibrillary tangles. Plaques are extracellular accumulations of Amyloid-β (Aβ), a proteolytic fragment of the Amyloid precursor protein, while the intracellular neurofibrillary tangle consists of abnormally phosphorylated, aggregated Tau. Similarly hyperphosphorylated and aggregated Tau is the primary neuropathologic manifestation of a less common group of neurodegenerative diseases including frontotemporal dementia and related disorders, known as "tauopathies." Genetic evidence for a causative role of Tau in neurodegeneration has been provided by the demonstration that dominant mutations in the *tau* gene cause frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17; Hong *et al.* 1998; Hutton *et al.* 1998; Spillantini *et al.* 1998). Although similar mutations have not been found in Alzheimer's disease, the appearance and anatomic distribution of neurofibrillary pathology correlates well with neuronal loss and cognitive dysfunction, suggesting that wild-type Tau may directly contribute to neuronal degeneration (Braak and Braak 1991; Arriagada *et al.* 1992).

The mechanism of Tau neurotoxicity in Alzheimer's disease and related disorders has been the subject of intensive investigation, and altered protein phosphorylation has been implicated as a major determinant of Tau toxicity (Lee *et al.* 2001). Tau protein purified from the brains of patients with Alzheimer's disease is hyperphosphorylated (Grundke-Iqbal *et al.* 1986; Ihara *et al.* 1986; Lee *et al.* 1991). In addition, antibodies recognizing selected Tau phosphoepitopes show specific staining of Tau from Alzheimer's disease brain tissue (Matsuo *et al.* 1994; Hasegawa *et al.* 1996; Jicha *et al.* 1997). In general, hyperphosphorylation decreases the affinity of Tau for microtubules and increases homotypic interactions, thus promoting aggregation (Gustke *et al.* 1992; Alonso *et al.* 2001). Several *tau* missense mutations associated with FTDP-17 have similar effects on microtubule binding and aggregation, suggesting that these changes might form the basis of Tau neurotoxicity (Hasegawa *et al.* 1998; Hong *et al.* 1998). Such observations have motivated extensive efforts to identify the kinases and phosphatases responsible for modulating Tau phosphorylation in Alzheimer's disease and related disorders. A number of candidates have been identified, including cyclin-dependent kinase 5 (CDK5), protein kinase A (PKA), glycogen synthase kinase 3 (GSK3), mitogen-activated protein kinase (MAPK), and protein phosphatase 2A (PP2A; reviewed in Lovestone and Reynolds 1997; Buee *et al.* 2000). In some cases, alterations in the expression, localization, or activity of candidate kinases have been observed in the brains of patients with Alzheimer's disease. However, experimental proof linking Tau hyperphosphorylation, or increased activity of particular kinases, to neurodegeneration *in vivo* has been complicated (Mattson 2001). In transgenic mice, expression of the CDK5 activator p25 (Ahlijanian *et al.* 2000), CDK5 together with p25 (Noble *et al.* 2003), a dominant negative form of PP2A (Kins *et al.* 2001), or GSK3 (Spittaels *et al.* 2000; Lucas *et al.* 2001) results in hyperphosphorylation of Tau, but effects on neurodegenerative cell death have been variable.

Drosophila models have been successfully developed

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for a number of neurodegenerative diseases, and these systems are now being exploited to dissect the genetic pathways underlying neurotoxicity (Muqit and Feany 2002). A major advantage of Drosophila as a model system is the ability to conduct unbiased genetic screens for enhancers and suppressors of neurodegeneration *in vivo*. This approach has been successfully applied to Drosophila models of the polyglutamine repeat disorders, which include Huntington's disease and spinocerebellar ataxia (Fernandez-Funez *et al.* 2000; Kazemi-Esfarjani and Benzer 2000). Such genetic screens, as well as candidate-based approaches, have revealed that mutations in heat-shock proteins and components of the ubiquitin/proteasome degradation pathway can modulate polyglutamine toxicity *in vivo* (Warrick *et al.* 1999; Chan *et al.* 2002). Molecular chaperones have been similarly implicated as modulators of neurodegeneration in a Drosophila model of Parkinson's disease (Auluck *et al.* 2002). These results suggest that the misfolding, impaired degradation, and abnormal aggregation of proteins are key determinants in the pathogenesis of neurodegenerative disease.

We have developed a Drosophila model of tauopathy that allows us to address the determinants of Tau toxicity *in vivo* (Wittmann *et al.* 2001). Expression of human *tau* in the Drosophila brain recapitulates several features of human tauopathies, including age-dependent neurodegeneration, early death, abnormally phosphorylated and folded Tau, and increased toxicity of disease-linked mutant *vs.* wild-type Tau. To elucidate the mechanisms of Tau neurotoxicity, we conducted a screen for genetic modifiers of Tau-induced neurodegeneration. Our results suggest that kinases and phosphatases are major determinants of Tau neurotoxicity *in vivo*. We also demonstrate that the molecular mechanisms mediating neuronal toxicity in tauopathies and polyglutamine diseases are largely distinct by comparing the activity of genetic modifiers in the Drosophila models of these diseases.

MATERIALS AND METHODS

Genetics: The upstream activating sequence (*UAS*)-*Tau*^{V337M} transgenic Drosophila line has been described previously (Wittmann *et al.* 2001). The enhancer-promoter (EP) strains and some mutant stocks were obtained from the Bloomington Drosophila Stock Center and from Exelixis. The following mutations and transgenic strains were used: *par-1*^{W3} and *UAS-par1* (Shulman *et al.* 2000); *UAS-stg* (Neufeld *et al.* 1998); *th*^{SL} and *UAS-th* (Lisi *et al.* 2000); *GMR-diap1* and *th*^{j5C8} (Hay *et al.* 1995); *stg*^{01235}, *fry*^{02240}, *cher*^{BG02734}, and *twe*^{k08310} (Spradling *et al.* 1999); *aop*^{1} (Rogge *et al.* 1995); *UAS-Atx2* (Satterfield *et al.* 2002); *UAS-wun2-myc* and *Df (2R)w73-1* (Starz-Gaiano *et al.* 2001); *UAS-aop*^{WT}, *GMR-yan*^{WT}, and *SEV-yan*^{ACT} (Rebay and Rubin 1995); *UAS-dally* (Jackson *et al.* 1997); *UAS-PKAmC* (Li *et al.* 1995); *UAS-PKAcF* (Kiger *et al.* 1999); *UAS-hep* (Boutros *et al.* 1998); *UAS-zw3* (Steitz *et al.* 1998); *UAS-cdk5-FLAG* (Connell-Crowley *et al.* 2000); *UAS-p35* (Ma and Haddad 1999); *Pros*^{1} (Smyth and Belote 1999); *UAS-Pros*^{1} (Schweisguth 1999); and *dfxr1*^{50M} (Zhang *et al.* 2001).

EP modifiers of the Tau-induced rough eye phenotype were selected on the basis of their ability to modify the phenotype of *UAS-Tau*^{V337M}*/+; GMR-GAL4/+* animals. Vials were coded numerically, and screeners did not have access to insertion site or molecular identity of relevant loci during the screening procedure. Candidate modifiers were also tested for their ability to modify the *UAS-Tau*^{V337M}*/+; GMR-GAL4/+*. Fly cultures and crosses were routinely carried out at 25°. The UAS/GAL4 expression system is temperature dependent, with increased expression at higher temperatures. In the case of candidate kinases that produced a rough eye when expressed with *GMR-GAL4* alone at 25°, additional crosses were performed at 17° (Figure 3). Effects of modifiers in a polyglutamine model were tested in the *UAS-SCA1-82/+; GMR-GAL4/+* genotype (Feany and Bender 2000).

Anatomic analyses: Expression was confirmed in EP lines by *in situ* hybridization to third instar larval central nervous system preparations with the EP element of interest *trans*-heterozygous to *GMR-GAL4* following a standard protocol (Wolff 2000). For electron microscopy, adult flies were dehydrated through a graded series of ethanol solutions, critical point dried, sputter coated, and examined with a scanning electron microscope.

RESULTS

A genetic screen for modifiers of Tau toxicity: Our Drosophila tauopathy model is based on the GAL4-UAS expression system, in which a human *tau* transgene downstream of a yeast UAS is controlled by driver lines that express the GAL4 transcriptional activator in particular spatial and temporal patterns (Brand and Perrimon 1993). When Tau expression was targeted to the eye using the *GMR-GAL4* driver line, adult flies showed a rough eye phenotype. In comparison to the normal fly eye (Figure 1A), expression of human Tau reduced the size of the eye and disrupted the regular array of lenses, reflecting disorganization of the underlying photoreceptor clusters, termed ommatidia (Figure 1B). The severity of the rough eye phenotype correlated well with the level of Tau expression, suggesting that the phenotype should be a sensitive substrate for second-site genetic modification. We chose a genotype, *UAS-tau*^{V337M}*/+; GMR-GAL4/+*, with a moderately rough eye (Figure 1B), to facilitate identification of both enhancers and suppressors of Tau toxicity. We screened insertion lines containing EP-transposable elements (Rorth *et al.* 1998). When the EP element is inserted proximal to a gene, and in the same orientation, it allows the ectopic expression of the locus under the control of GAL4. Alternatively, when inserted in the reverse orientation, the EP element often inactivates expression of the gene. Thus, our EP screen had the potential to identify both gain-of-function and loss-of-function modifiers of Tau toxicity.

We carried out an F_1 screen of an established collection of 2276 EP transposable elements by crossing flies expressing human Tau in the eye to individual EP insertion lines and examining the progeny for dominant enhancement or suppression of the Tau-induced rough eye phenotype. Suppressors of Tau toxicity in the eye restored the eye to normal size and significantly ameliorated the ommatidial irregularity (Figure 1, C–E). In contrast, enhancers of the Tau rough eye phenotype

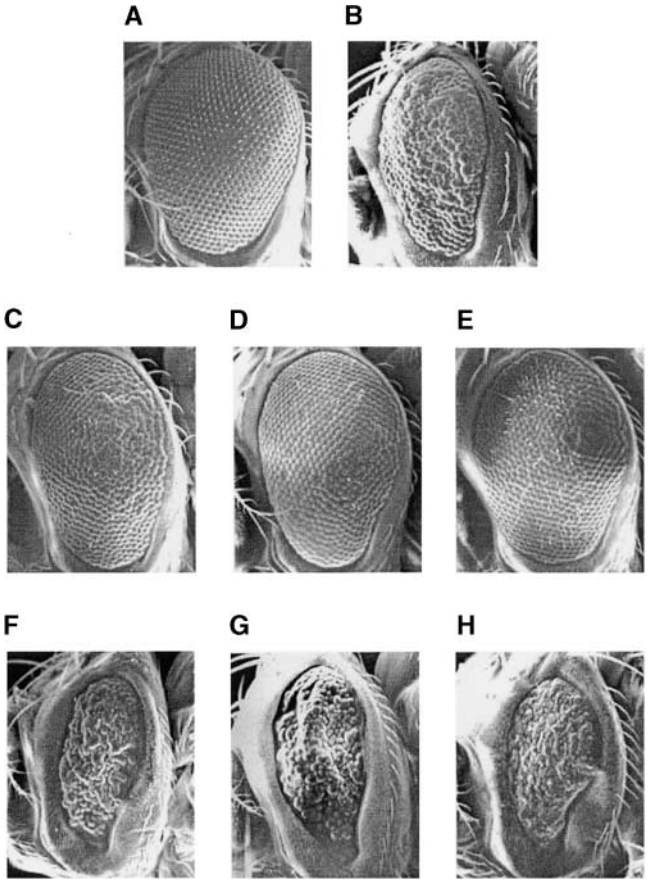

FIGURE 1.—Genetic modifiers suppress or enhance Tau toxicity in the retina. (A) Wild-type eye showing regular arrangement of ommatidia. (B) Moderate rough eye produced by expression of Tau^{V337M} with *GMR-GAL4*. (C–E) Rescue of Tau retinal toxicity by (C) *EP(3)3518*, (D) *UAS-par1*, and (E) *UAS-stg*. (F–H) Enhancement of Tau toxicity by (F) *EP(2)2504*, (G) *EP(3)3319*, and (H) *EP(3)3403*.

further reduced the eye in size and produced increased ommatidial irregularity and fusion of the overlying lenses (Figure 1, F–H). The quality and strength of the effects shown in Figure 1 are representative of the modifiers recovered in our screen. All candidate modifiers were subjected to a series of validation tests. We first generated precise excisions for each EP line to demonstrate reversion of the modifier activity and pursued only those EP lines that showed significant enhancement or suppression of Tau toxicity relative to background chromosome effects. Next, all of the candidate enhancers were crossed to *GMR-GAL4*. We discarded any lines that caused a moderate or severe rough eye phenotype on their own. We did retain a limited subgroup of modifiers [*EP(2)2028*, *EP(2)2437*, *EP(3)3517*, and *EP(3)3559*] that produced a very mild rough eye in combination with *GMR-GAL4*. However, expressing Tau in combination with these EP elements produced a severe rough eye, consistent with synergistic enhancement by the EP elements.

The EP insertion position and orientation were determined initially using the online database resources of FlyBase and were confirmed as detailed below. We pursued further only modifiers for which a single locus was unambiguously affected by the EP insertion. In nearly all cases the EP-transposable element was inserted directly within the candidate transcription unit or within 100 bp of its start site. In three cases, *EP(3)1072*, *EP(3)3569*, and *EP(2)2190*, the elements were inserted within 1 kb of the transcription start, with no other potential loci in the immediate vicinity. In several instances, multiple insertions were recovered, affecting the same locus (see Table 1). In one notable case, the insertions *EP(3)3569* and *EP(3)1072* were independently recovered as a suppressor and enhancer, respectively, and were inserted at the same genomic position but in opposite orientations, demonstrating both gain-of-function and loss-of-function effects. Where the EP element was inserted proximal to and in the same orientation as a candidate gene (19/24 cases), we could often validate overexpression of the predicted locus. For many loci, previously published UAS transgenic stocks were obtained and tested for modifier activity. In several other cases, we performed mRNA *in situ* hybridization to demonstrate enhanced expression of the locus under the control of *GMR-GAL4*. Where possible we tested mutant alleles of the candidate loci as Tau modifiers. In three cases (see Table 1), analysis of mutant alleles revealed that gain of function and loss of function of the same locus modified Tau neurotoxicity in opposite directions.

In the remaining cases (6/24), where we were unable to validate the affected loci with multiple insertions, UAS transgenes, mRNA *in situ* hybridization, or loss-of-function alleles, inverse PCR and sequencing were performed to confirm the EP-insertion position. Finally, we used Western blot analysis on all candidate suppressors to demonstrate that none simply reduced Tau expression (data not shown). The resulting 8 suppressors and 16 enhancers of Tau toxicity that fulfilled all validation criteria are presented in Table 1 (representative examples are shown in Figure 1, C–H). Table 1 also shows the results of the validation tests for each modifier.

Kinases and phosphatases are the major class of Tau modifiers: The largest functional class of modifiers encoded kinases or phosphatases, including Drosophila homologs of several enzymes known to phosphorylate or dephosphorylate Tau (Table 1). *EP(2)0899*, a Tau suppressor, is predicted to activate expression of the fly ortholog of the microtubule affinity-regulating kinase (MARK)/PAR-1 serine/threonine kinase. Suppression of the Tau rough eye phenotype by increasing PAR-1 expression was confirmed using a *UAS-par1* transgene (Table 1, Figure 1D).

We also identified subunits of the known Tau phosphatases PP1 and PP2A. *EP(3)3518* was identified as a suppressor (Table 1, Figure 1C) and is predicted to activate expression of a regulatory subunit of PP1. We confirmed overexpression by mRNA *in situ* hybridization. *EP(3)3559*, previously shown to activate expression

TABLE 1

Enhancers and suppressors of Tau toxicity

Gene	Mammalian homolog/function	EP insertion	Cytological location	Modification[a]	EP orientation/ overexpression[b]	Alleles tested/ modification[c]
			Protein kinases/phosphatases			
par-1	MARK serine/ threonine kinase	*EP(2)0899*[e]	56D	Su	S/U	$par\text{-}1^{W3}$/none
CG14217	Tao1 serine/ threonine kinase	*EP(X)1455*	18D	En	S/I	NA
center divider	TESK1 serine/ threonine kinase	*EP(3)3319*	91E	En	S	NA
string	CDC25 phosphatase	*EP(2)1213*[d]	99A	Su	S/U	stg^{01235}/none
twine	CDC25 phosphatase	*EP(2)0613*	35F	Su	S	twe^{k08310}/none
CG9238	PP1 phosphatase subunit	*EP(3)3518*	70E	Su	S/I	NA
CG5643	PP2A phosphatase subunit B	*EP(3)3559*	98A	En	S/I[e]	NA
			Apoptosis			
thread	IAP1 apoptosis inhibitor	*EP(3)3308*	72D	Su	S/U	th^{j5C8}/En; th^{SL}/En
CG9025	Fem1 apoptosis activator	*EP(2)2504*	57B	En	O	NA
			Cytoskeleton			
orbit	Microtubule-associated protein	*EP(3)3403*	78C	En	S/I	NA
dfxr1	Fragile-X	*EP(3)3517*	85F	En	S/U[f]	$dfxr1^{50M}$/none
cheerio	Filamin	*EP(3)3715*	89E	En	S/W[g]	$cher^{BG02734}$/none
			Miscellaneous			
CG5166	Ataxin-2	*EP(3)3145*	88F	En	S/U	NA
wunen	Phosphatidic acid phosphatase	*EP(2)2208*	45D	Su	S/U	*Df(2R)w73-1*/En
yan/aop	Transcription factor	*EP(2)2500*	22D	En	S/U	aop^{1}/none
dally	Glypican	*EP(3)0581*[d]	66E	En	S/U	$dally^{P16852A}$/none
CG8487	Sec7 GTPase exchange factor	*EP(2)2028*	59C	En	S/I[e]	NA
CG13610	Organic cation transporter	*EP(3)3569*[d]	95F	Su	S	NA
CG13610	Organic cation transporter	*EP(3)1072*	95F	En	O	NA
			Novel			
CG3735	Novel	*EP(2)2311*	60B	Su	S	NA
CG7231	Novel	*EP(2)2510*	28D	En	O	NA
CG10927	Novel	*EP(2)2190*	55E	En	O	NA
furry	Novel	*EP(3)0326*	67C	En	S	fry^{02240}/Su
SD02913	Novel	*EP(2)2437*[d]	53E	En	O	NA

[a] En, enhancer; Su, suppressor.

[b] Orientation of EP element relative to transcription unit and validation of overexpression, if applicable. S, same; O, opposite; U, UAS-transgene; I, mRNA *in situ* hybridization; W, Western blot.

[c] Loss-of-function alleles of candidate genes were tested for modifier activity. NA, alleles not available.

[d] Multiple independent insertions were identified (see text), but only one is listed, for simplicity.

[e] mRNA *in situ* performed by Kraut *et al.* (2001).

[f] UAS confirmation of expression by Zhang *et al.* (2001).

[g] Western blot confirmation of expression by Guo *et al.* (2000).

of a PP2A regulatory subunit (Kraut *et al.* 2001), was identified as a Tau enhancer.

In addition to MARK/PAR-1, two additional serine/threonine kinases were recovered in our screen. Both of these proteins have well-conserved mammalian homologs and behaved as enhancers of Tau toxicity.

EP(3)3319 (Figure 1G) is predicted to activate expression of the center divider kinase (Matthews and Crews 1999), and *EP(X)1455* activates expression of CG14217, a Drosophila homolog of the STE20-related kinase, Tao1 (Hutchison *et al.* 1998).

Our screen also identified two Drosophila homologs of the CDC25 phosphatase, *string* and *twine*, as suppressors of Tau. Three activating insertions in *string*, *EP(2)1213*, *EP(2)3426*, and *EP(2)3432*, were recovered independently as Tau suppressors. We confirmed the ability of String to suppress Tau toxicity using a *UAS-string* transgene (Figure 1E). Twine was identified as a single activating insertion, *EP(2)613*.

Genetic modifiers implicate apoptosis in Tau toxicity: In addition to kinases and phosphatases, we identified a number of other genetic modifiers that address the mechanism of Tau toxicity. Two of our enhancers have been implicated in apoptotic regulation. Thread (Th), a Drosophila homolog of the inhibitor of apoptosis proteins (IAPs), binds and inactivates pro-apoptotic caspases (Hay *et al.* 1995; Lisi *et al.* 2000). *EP(3)3308*, a Tau suppressor, is predicted to activate expression of *th*. We have confirmed that overexpression of Th suppresses Tau toxicity, using *UAS-th* and *GMR-th* transgenes (Table 1). Reciprocally, a *thread* loss-of-function allele, th^{jC58}, and a dominant negative allele, th^{SL}, both enhanced the tau rough eye. The other apoptosis-related modifier that we identified, *EP(2)2504* (Figure 1F), is predicted to express a homolog of the *C. elegans* Fem-1 protein (Doniach and Hodgkin 1984). Fem-1 is a substrate for the apoptotic caspase CED-3, binds directly to the apoptotic regulator CED-4, and modulates apoptosis in cultured cells (Chan *et al.* 2000).

Novel mediators of Tau toxicity: Two of our modifiers, *EP(3)3145* and *EP(3)3517*, alter the expression of Drosophila homologs of genes mutated in human neurological diseases (Table 1). *EP(3)3145* increases the expression of an Ataxin-2 homolog. *EP(3)3517* activates expression of the Drosophila homolog of the Fragile-X mental retardation protein (Fmr1). An inactivating trinucleotide repeat expansion in human FMRP causes the most common inherited form of mental retardation (Verkerk *et al.* 1991). In flies, Fmr1 represses translation of the microtubule-associated protein Futsch, a Drosophila Map1b homolog (Zhang *et al.* 2001). Our screen also identified a second protein implicated in microtubule function. Expression of the Drosophila microtubule-associated protein Orbit (Inoue *et al.* 2000; Lemos *et al.* 2000) via the *EP(3)3403* element enhanced Tau toxicity. In addition, we have identified *cheerio*, a Drosophila ortholog of the actin-binding protein, Filamin, as a Tau enhancer (Sokol and Cooley 1999). Last, our screen has identified several novel, conserved genes. This modifier class includes the suppressor, *EP(2)2311*, and the enhancers, *EP(2)2510*, *EP(2)2190*, *EP(3)326*, and *EP(2)2437*.

Known Tau kinases modulate Tau toxicity *in vivo*: Given the number of kinases and phosphatases identified by our screen, we tested if other kinases known to phosphorylate Tau *in vitro* could modify Tau toxicity *in vivo*. Members of the MAPK superfamily phosphorylate Tau in an N-terminal proline-rich domain. In particular, the c-jun N-terminal kinase (JNK) and stress-activated protein kinase subfamily has been implicated in pathological Tau phosphorylation (Goedert *et al.* 1997; Reynolds *et al.* 2000; Zhu *et al.* 2000, 2001). Expression of Hemipterous, the Drosophila homolog of the JNK-kinase, activates the JNK pathway in the eye (Boutros *et al.* 1998) and enhanced Tau toxicity (Figure 2). Coexpression of Hemipterous with Tau decreased eye size, increased surface roughness, and induced the formation of necrotic black patches, as compared with control flies expressing Tau alone (compare Figure 2A with 2B, arrow shows necrotic spot). Expression of Hemipterous alone in the eye under the control of *GMR-GAL4* did not affect eye morphology.

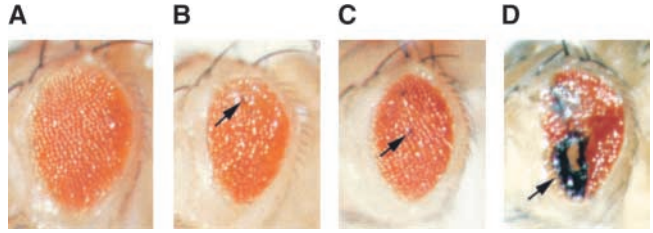

Figure 2.—Known Tau kinases enhance Tau toxicity *in vivo* with reduction in the size of the eye and formation of necrotic foci (arrows). (A) Moderate rough eye phenotype produced by the expression of Tau^{V337M} using *GMR-GAL4*. (B) The JNK kinase Hemipterous enhances the Tau^{V337M} rough eye phenotype. (C) Protein kinase A expression enhances the Tau^{V337M} rough eye phenotype. (D) Expression of both CDK5 and its activator p35 together enhances the rough eye produced by Tau^{V337M}.

Like the MARK kinase, PKA can phosphorylate residues within the Tau microtubule-binding repeats (Ser262, Ser324, and Ser356) and can additionally mediate phosphorylation within a flanking domain at Ser214 (Zheng-Fischhofer *et al.* 1998; Schneider *et al.* 1999). To test the effect of PKA, we used a constitutively active version of murine PKA that had no effect on the eye when expressed alone. Expression of active mouse PKA enhanced the toxicity of human Tau in the retina as seen by the reduction in the size of the eye and the formation of necrotic foci (Figure 2C, arrow). In addition, a constitutively active version of Drosophila PKA strongly enhanced the rough eye caused by expressing human Tau in photoreceptor cells of the retina using *elav-GAL4* (data not shown).

The CDC2-related kinase, CDK5, has received significant attention as a potential mediator of Tau phosphorylation in disease. The CDK5 regulatory subunit, p35, is abnormally cleaved to p25 in Alzheimer's brain, and the resulting p25/CDK5 complex has enhanced Tau kinase activity (Baumann *et al.* 1993; Patrick *et al.*

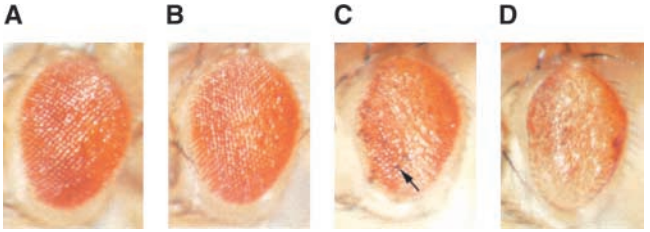

Figure 3.—Analysis of Tau modifier effects on polyglutamine toxicity. (A) Expression of expanded SCA1 produces a mildly rough and depigmented eye. (B) Most Tau modifiers, including the center divider kinase *EP(3)3319*, do not modify polyglutamine toxicity. (C and D) Two polyglutamine enhancers also enhance Tau toxicity, including *EP(2)2510* (C, arrow indicates a blackened, necrotic patch in the retina) and the Drosophila Ataxin-2 homolog *EP(3)3145* (D).

1999). When tested individually, neither CDK5 nor p35 expression modified the Tau rough eye phenotype. However, coexpression of both CDK5 and p35 potently enhanced the rough eye produced by Tau (Figure 2D). Although expressing both CDK5 and p35 in the eye using *GMR-GAL4* driver produced a mild rough eye, coexpression of CDK5/p35 with human Tau resulted in synergistic retinal toxicity including large necrotic patches (Figure 2D, arrow) and sunken areas representing loss of underlying retinal tissue (Figure 2D). In addition, when crosses were carried out at 18° to decrease the activity of the GAL4-UAS expression system, CDK5/p35 expression no longer caused a rough eye alone, but still markedly enhanced the Tau rough eye phenotype (data not shown).

Most Tau modifiers do not affect polyglutamine toxicity: Although neurodegenerative disorders like Alzheimer's disease, Parkinson's disease, and Huntington's disease have distinct clinical manifestations, they have common features that suggest they might share fundamentally similar mechanisms of pathogenesis (Trojanowski and Lee 2000). We have now used Drosophila genetics to investigate the relationship between the toxicity of Tau and polyglutamine repeat-containing proteins.

We first tested the activity of all of our Tau modifiers in a polyglutamine disease model, spinocerebellar ataxia type 1 (SCA1). Expression of a human *SCA1* transgene with an expanded polyglutamine track produces a moderately rough and depigmented eye (Figure 3A; Feany and Bender 2000; Fernandez-Funez *et al.* 2000). A total of 22 of 24 Tau modifiers had no effect on the eye phenotype produced by expression of mutant human SCA1 (Figure 3B). Significantly, all of the kinases and phosphatases that potently affected Tau toxicity failed to modify the SCA1-induced eye phenotype, suggesting that this functional group is not a determinant of polyglutamine toxicity [*EP(3)3319* is shown as an example in Figure 3B]. Two Tau modifiers did enhance SCA1 toxicity: *EP(2)2510* (Figure 3C) and *EP(3)3145* (Figure 3D). Both modifiers enhanced the loss of eye pigmentation and induced necrotic black dots (Figure 3C, arrow). Interestingly, *EP(3)3145* appears to activate expression of a Drosophila homolog of Ataxin-2. In contrast to the results of our Tau modifier screen, previous Drosophila genetic screens for modifiers of polyglutamine toxicity have not identified kinases and phosphatases. A number of studies have instead implicated heat-shock proteins, chaperones, and components of the ubiquitin-proteasome pathway as key determinants of polyglutamine toxicity (Warrick *et al.* 1999; Fernandez-Funez *et al.* 2000; Kazemi-Esfarjani and Benzer 2000; Chan *et al.* 2002). In contrast, none of the Tau modifiers identified in our forward genetic screen directly controls protein folding or degradation, suggesting a distinct mechanism of toxicity. We have further examined the relationship between Tau and polyglutamine toxicity by directly testing all previously identified modifiers of polyglutamine toxicity that were available to us and that did not cause a rough eye in combination with *GMR-GAL4* in our tauopathy model (Table 2). Consistent with the results above, all of these modifiers, including numerous heat-shock proteins, chaperones, and ubiquitin pathway components, failed to modify Tau toxicity.

DISCUSSION

Multiple lines of evidence support a central role for Tau in the pathogenesis of Alzheimer's and related neurodegenerative diseases. Most significantly, neurofibrillary tangle pathology correlates well with neuronal loss and cognitive dysfunction (Braak and Braak 1991; Arriagada *et al.* 1992), and mutations in the *tau* gene cause the familial neurodegenerative syndrome, FTDP-17 (Hong *et al.* 1998; Hutton *et al.* 1998; Spillantini *et al.* 1998). Here we report a genetic screen for modifiers of Tau neurotoxicity. Our screen has identified 16 enhancers and 8 suppressors of Tau toxicity. Nearly one-third of these modifiers encode protein kinases and phosphatases, the largest single functional class recovered. Several of these modifiers, including the MARK kinase and the PP1 and PP2A phosphatases, have been previously shown to phosphorylate or dephosphorylate Tau *in vitro* (Yamamoto *et al.* 1988; Hasegawa *et al.* 1992; Goedert *et al.* 1995; Liao *et al.* 1998; Sontag *et al.* 1999). We further demonstrate that several known Tau kinases, including CDK5, PKA, and the JNK pathway, also enhance Tau toxicity *in vivo*. The Tau kinase GSK3β has also been shown to enhance Tau toxicity in Drosophila (Jackson *et al.* 2002).

Many of the kinases and phosphatases that control Tau neurotoxicity in transgenic flies have been previously implicated in the pathogenesis of Alzheimer's disease on the basis of alterations in localization or activity in postmortem brain samples from patients. The

TABLE 2

Modifiers of polyglutamine toxicity tested for Tau modification

Modifier	Locus	Polyglutamine modification[a]	Tau modification
l(3)05634	*Ubi63E*	En (SCA1)	—
l(3)neo55	*hsr-ω*	En (SCA1)	—
EP(3)0674	*UbcD1*	En (SCA1)	—
EP(X)1303	*dUbc-E2H*	En (SCA1)	—
EP(2)2231	*Gst3*	Su (SCA1)	—
EP(2)2417	*nup44A*	Su (SCA1)	—
EP(3)3623	*mub*	Su (SCA1)	—
EP(3)3461	*pum*	En (SCA1)	—
EP(3)3378	*cpo*	En (SCA1)	—
EP(2)0866	*Sin3A*	En (SCA1)	—
l(2)08269	*Sin3A*	Su (HD)	—
EP(3)3672	*Rpd3*	En (SCA1)	—
EP(2)2300	*dSir2*	En (SCA1)	—
EP(3)3463	*tara*	En (SCA1)	—
Pros^{1}	*Pros26*	En (SCA1)	—
UAS- Pros^{1}	*Pros26*	En (SBMA)	—
UAS-hsc4DN	*hsc4*	En (SCA3)	—
UAS-hsp70	*hsp70*	Su (SCA3)	—

[a] Modifiers were described in models of spinocerebellar ataxia type 1 (Fernandez-Funez *et al.* 2000), spinocerebellar ataxia type 3 (Warrick *et al.* 1999), Huntington's disease (Steffan *et al.* 2001), and spinobulbar muscular atrophy (Chan *et al.* 2002).

MARK kinase and activated JNK colocalize tightly with neurofibrillary tangles (Chin *et al.* 2000; Zhu *et al.* 2000, 2001). PP2A mRNA levels are abnormally decreased in Alzheimer's disease brains (Vogelsberg-Ragaglia *et al.* 2001). Similarly, the expression and activity of CDC25 and its substrate, CDC2, have both been found to be dysregulated in Alzheimer's brain (Vincent *et al.* 1997, 2001; Ding *et al.* 2000). The CDK5 regulatory subunit, p35, can be abnormally cleaved to p25 in Alzheimer's, resulting in constitutive activity of CDK5 (Patrick *et al.* 1999). Our finding that these kinases and phosphatases, which have altered distributions and/or activities in disease states, can also control Tau toxicity *in vivo* supports the identification of these enzymes as key therapeutic targets in Alzheimer's disease and related disorders.

We have also identified two additional conserved serine/threonine kinases as Tau modifiers. Activating expression of either the center divider kinase or a Drosophila homolog of the Tao1 kinase enhanced Tau toxicity. The center divider kinase is expressed in the developing Drosophila nervous system and has a well-conserved mammalian homolog (Matthews and Crews 1999). Tao1 is highly expressed in the rat brain (Hutchison *et al.* 1998). These kinases represent attractive candidates for involvement in the pathogenesis of Alzheimer's disease and related disorders. In future studies, it will be important to determine whether these kinases can directly phosphorylate Tau and whether the distribution or activity of the human homologs is altered in disease states. These enzymes will also be candidates for testing in vertebrate tauopathy models.

Tau isolated from the brains of patients dying with Alzheimer's disease and related disorders characterized by abnormal Tau deposition is abnormally hyperphosphorylated, and many Tau phosphoepitopes are specifically associated with disease in the adult brain. These observations have long fueled speculation that phosphorylation of Tau determines neurotoxicity. However, direct experimental demonstration that phosphorylation controls neurodegenerative cell death *in vivo* has been complicated (Mattson 2001). Overexpression of GSK3β in mice induces Tau hyperphosphorylation, but altered phosphorylation has been correlated with both increases (Lucas *et al.* 2001) and decreases (Spittaels *et al.* 2000) in neurodegeneration. Expression of CDK5 with its activator p25 enhances Tau phosphorylation and aggregation in transgenic mice expressing mutant human Tau (Noble *et al.* 2003). We have previously shown that, as in human disease, transgenic human Tau is abnormally phosphorylated in the Drosophila brain and that the development of disease-linked Tau phosphoepitopes correlates both spatially and temporally with neuronal degeneration (Wittmann *et al.* 2001). Here, we show that kinases and phosphatases are the major determinants of neurodegeneration in our Drosophila model, including several enzymes known to directly phosphorylate or dephosphorylate Tau. These results strongly support a link between Tau phosphorylation and neurotoxicity *in vivo*.

How might Tau phosphorylation alter Tau toxicity? Overall, our results support a model in which increased Tau phosphorylation correlates with increased toxicity. For six of the seven kinase modifiers, increasing kinase expression enhances both Tau phosphorylation and toxicity *in vivo*. A number of *in vitro* studies have demonstrated that hyperphosphorylation decreases the affinity of Tau for microtubules and increases homotypic interactions, thus potentially favoring cytosolic accumulation and aggregation *in vivo* (Gustke *et al.* 1992; Alonso *et al.* 2001). Many of the mutations in *tau* that cause FTDP-17 similarly reduce the interaction of Tau with microtubules and promote Tau oligomerization (Hasegawa *et al.* 1998; Hong *et al.* 1998). Thus, decreased microtubule affinity, increased aggregation, or both may enhance the neurotoxicity of Tau in flies. Interestingly, the one exception to our finding that increasing kinase expression correlates with enhanced Tau toxicity is MARK/PAR-1, which behaves as a genetic suppressor and is known to phosphorylate Tau at Ser262 (Drewes *et al.* 1997). While phosphorylation of this residue within the microtubule-binding domain abolishes the binding of Tau to microtubules *in vitro* and *in vivo*, phosphorylation at this site was also found to strongly inhibit aggregation (Schneider *et al.* 1999). Although significant numbers of large filamentous Tau aggregates are not present in

flies expressing human Tau (Wittmann *et al.* 2001), our identification of MARK/PAR-1 as a suppressor may be consistent with the possibility of a smaller, perhaps protofilamentous, toxic aggregate.

A number of neurodegenerative diseases have now been modeled in Drosophila, including Parkinson's disease (Feany and Bender 2000), tauopathies (Wittmann *et al.* 2001), Huntington's disease (Jackson *et al.* 1998), and spinocerebellar ataxias (Warrick *et al.* 1998; Fernandez-Funez *et al.* 2000). These and related models have been used to identify genetic modifiers (Warrick *et al.* 1999; Fernandez-Funez *et al.* 2000; Kazemi-Esfarjani and Benzer 2000; Steffan *et al.* 2001; Chan *et al.* 2002). The availability of multiple Drosophila models of neurodegenerative diseases, and a growing collection of genetic modifiers, allows us to compare cellular pathways controlling neurodegenerative cell death. Similarities in the clinical and neuropathologic features of the cognate human neurodegenerative diseases have suggested that the disorders may share similar mechanisms of pathogenesis related to abnormal protein folding and aggregation (Trojanowski and Lee 2000).

In contrast, our evidence supports distinct mechanisms of toxicity in polyglutamine disorders and tauopathies. First, our Tau screen identified a completely nonoverlapping group of modifiers compared with previous screens for polyglutamine modifiers. In at least one case, the identical collection of EP elements was screened (Fernandez-Funez *et al.* 2000). The largest class of polyglutamine modifiers recovered to date consists of chaperones and ubiquitin-proteasome pathway components. We have not identified any of these genes in our screen. Instead, the largest single class of Tau modifiers includes kinases and phosphatases. Second, we have tested all of our Tau modifiers in a Drosophila SCA1 model. Most show no effect on polyglutamine toxicity. Third, we have also tested most of the published modifiers of polyglutamine toxicity in our tauopathy model and found that none affect Tau toxicity. These results suggest that Tau and polyglutamine toxicities in Drosophila are mostly controlled by distinct sets of genes with roles in different biological processes. Thus, diverse therapeutic approaches may be required in neurodegenerative diseases that seemingly share key similarities.

Although the majority of Tau and polyglutamine modifiers define nonoverlapping sets, we did identify exceptions. Two EP enhancers from our Tau screen also enhanced SCA1. One encodes a novel protein, and the other activates expression of a Drosophila Ataxin-2 homolog. Expansion of a polyglutamine tract in human Ataxin-2 produces a spinocerebellar ataxia with clinical and neuropathological similarities to SCA1 (De Girolami and Feany 2001). These shared modifiers may define convergent pathways of toxicity.

In conclusion, an analysis of modifiers recovered in our screen suggests a genetic pathway for Tau toxicity in human disease. We propose that kinases and phosphatases play a critical early role in disease pathogenesis, perhaps by modulating the affinity of Tau for microtubules and thereby increasing the cytoplasmic Tau fraction. Elevated levels of free Tau favor the formation of an abnormally folded, toxic Tau species. The next step in this cascade remains undefined; however, our genetic modifiers may identify some of the relevant molecular pathways. In particular, our screen identified several novel, highly conserved proteins that may transduce the toxic effects of abnormal Tau. Our recovery of multiple modifiers that function in cytoskeletal regulation may implicate the neuronal cytoskeleton as a possible subcellular target. Finally, our findings that genetic modifiers related to apoptosis also influence Tau neurotoxicity correlate with other findings that implicate apoptosis as the end pathway of neurodegenerative cell death in Alzheimer's disease and tauopathies (Roth 2001; Jackson *et al.* 2002). Our genetic findings highlight targets for possible therapeutic intervention. In addition, determinants of Tau toxicity in Drosophila suggest candidate loci for familial neurodegenerative syndromes as well as potential modifier genes in Alzheimer's disease and related disorders.

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