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S/P and T/P Phosphorylation Is Critical for Tau Neurotoxicity in *Drosophila*

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The microtubule-associated protein tau is hyperphosphorylated abnormally in AD and related neurodegenerative disorders. Many phospho epitopes created by proline directed kinases (SP/TP sites) show relative specificity for disease states. To test whether phosphorylation at the disease-associated SP/TP sites affects tau toxicity in vivo, we expressed a form of tau in *Drosophila* in which all SP/TP sites are mutated to alanine. We find that blocking phosphorylation at SP/TP motifs markedly reduces tau toxicity in vivo. Using phosphorylation-specific antibodies, we identify a positive correlation between increased phosphorylation at disease-associated sites and neurotoxicity. We use the phosphorylation-incompetent version of tau to show that kinase and phosphatase modifiers of tau neurotoxicity, including cdk5/p35, the JNK kinase hemipterous and PP2A act via SP/TP phosphorylation sites. We provide direct evidence in an animal model system to support the role of phosphorylation at SP/TP sites in playing a critical role in tau neurotoxicity.

Key words: Alzheimer's disease; kinase; neurodegeneration; phosphorylation; tauopathy

Tau is a microtubule-associated protein implicated in the pathogenesis of Alzheimer's disease (AD) and related disorders. Mutations in the tau gene cause autosomal dominant frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (Hong et al., 1998; Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). In addition to FTDP-17, several other disorders, including AD, are characterized pathologically by intracellular neurofibrillary inclusions formed of hyperphosphorylated and aggregated tau protein. These diseases are collectively known as tauopathies (Feany and Dickson, 1996; Lee et al., 2001). In AD, accumulation of hyperphosphorylated tau in neurofibrillary tangles (NFTs) correlates with disease progression, severity, and cognitive impairment (Grundke-Iqbal et al., 1986; Ihara et al., 1986; Braak and Braak, 1991; Dickson et al., 1995). Although tau mutations have not been identified in AD and other sporadic tauopathies, tau likely contributes to neuronal degeneration in these disorders, possibly through aberrant phosphorylation and aggregation.

Tau is phosphorylated by a number of kinases (Buee et al., 2000; Gong et al., 2005). The proline-directed protein kinases phosphorylate tau on serine (S) or threonine (T) residues that are immediately flanked by proline (P), sequences known as SP or TP motifs. Proline-directed protein kinases include glycogen synthase kinase 3 (GSK3), mitogen-activated protein kinase, extracellular signal regulated kinase (ERK1/2), Jun N-terminal kinase (JNK1), and the cyclin-dependent kinases cdk5 and cdc2. The proline-directed protein kinases have received a great deal of attention because phospho epitopes at SP/TP sites are recognized by antibodies that preferentially detect tau in brain tissue from patients with tauopathies. Other tau kinases include cyclic-AMP-dependent protein kinase A (PKA), microtubule-affinity regulating kinase (MARK), protein kinase C, calcium/calmodulin dependent protein kinase II, and casein kinase II. In vitro studies suggest that phosphorylation by these kinases controls the affinity of tau for microtubules and may prime tau for subsequent phosphorylation by other kinases (Biernat et al., 1993; Zheng-Fischhofer et al., 1998). Tau is dephosphorylated by protein phosphatase-1, -2A (PP2A), -2B (calcineurin), and -2C, although PP2A is likely the dominant tau phosphatase in vivo (Sontag et al., 1996).

The accumulation of hyperphosphorylated tau in disease states implicates phosphorylation in tau neurotoxicity. In addition, a number of experiments have been carried out in animal models to regulate the phosphorylation state of tau by altering kinase or phosphatase activity and then determine the effects on toxicity. Many of these studies

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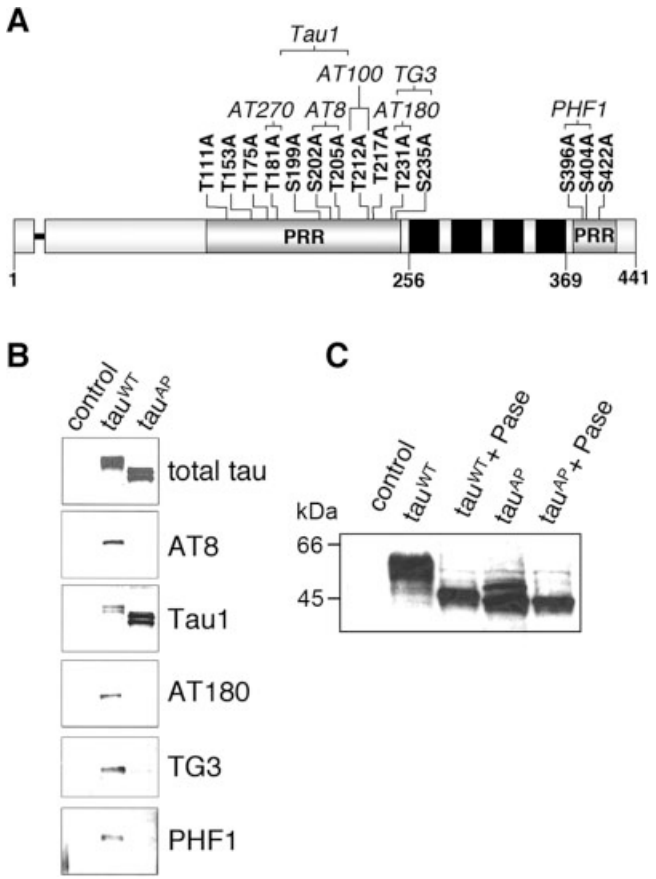

Fig. 1. Phosphorylation of wild-type and mutant human tau in *Drosophila*. **A:** Schematic of point mutations and antibody epitopes of tau^{AP}. The 14 point mutations from serine (S) or threonine (T) to alanine (A) are located in either of two proline rich regions (PRR). The epitopes recognized by phosphorylation-dependent antibodies are shown. **B:** Phosphorylation-dependent antibodies AT8 (specific for pS202/pT205), AT180 (pT231), TG3 (pT231/pS235), and PHF1 (PS396/pS404) recognize wild-type human tau (tau^{WT}) but not tau^{AP} expressed in flies. The Tau1 antibody preferentially recognizes unphosphorylated tau between amino acids 189–207. **C:** Wild-type and mutant forms of human tau are phosphorylated in flies. Phosphatase treatment (Pase) of both tau^{WT} and tau^{AP} alters the migration of tau. Control is *GMR-GAL4/+*.

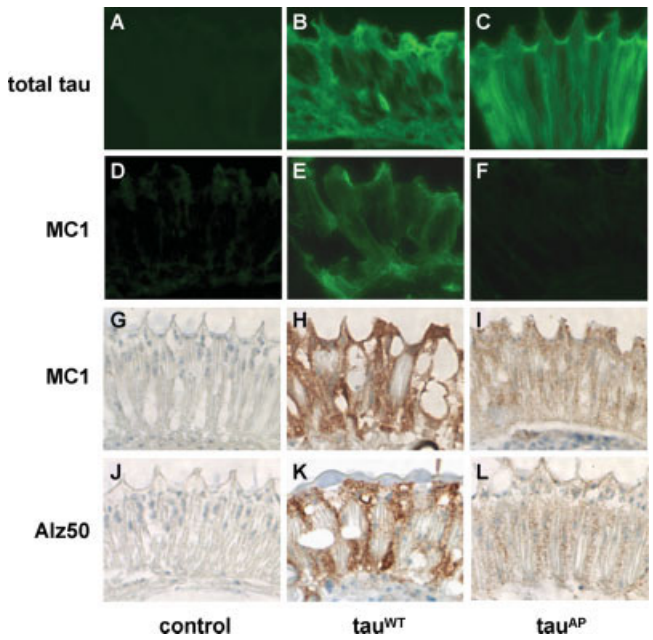

Fig. 2. Abolishing S/P and T/P phosphorylation alters the conformation of tau. The antibodies MC1 and Alz50 recognize abnormal conformations of tau associated with disease states. No total tau, MC1, or Alz50 immunoreactivity is detected in controls (**A**,**D**,**G**,**J**; *GMR-GAL4/+*). Staining with a polyclonal antibody for total tau detects similar levels of tau protein in the retinas of tau^{WT} and tau^{AP}-expressing flies (**B**,**C**). The MC1 epitope is detected by immunofluorescence in flies expressing tau^{WT} (**E**), but not tau^{AP} (**F**) in the retina. A higher sensitivity histochemical detection method confirms clear MC1 (**H**) and Alz50 (**K**) immunoreactivity in flies expressing tau^{WT}, and shows decreased MC1 (**I**) and Alz50 (**L**) immunoreactivity in flies expressing tau^{AP}.

support the role of phosphorylation in promoting neurotoxicity (Ahlijanian et al., 2000; Lucas et al., 2001; Jackson et al., 2002; Cruz et al., 2003; Noble et al., 2003, 2005; Nishimura et al., 2004; Le Corre et al., 2006). However, results from other studies have been more variable (Spittaels et al., 2000; Kins et al., 2001; Bian et al., 2002). The disparate results may be due, at least in part, to the ability of many kinases and phosphatases to function in multiple pathways. The fact that individual kinases and phosphatases may be involved in the regulation of multiple substrates in vivo underscores the need to validate directly the role of tau phosphorylation in vivo.

To address directly whether phosphorylation at the SP/TP disease-associated sites plays a role in tau toxicity in vivo, we used a form of tau in which all of the SP/TP sites have been mutated to alanine (tau^{AP}). We report that phosphorylation at SP/TP sites is a critical mediator of tau toxicity in vivo.

MATERIALS AND METHODS

Constructs, Genetics, and Stocks

The *UAS-tau^{WT}* transgenic *Drosophila* line has been described previously (Wittmann et al., 2001). The *UAS-tau^{AP}* transgenic line was derived by cloning the tau23/AP construct (Biernat and Mandelkow, 1999) plus the second microtubule (MT) binding domain into the pUAST vector. Like *UAS-tau^{WT}*, *UAS-tau^{AP}* therefore contains all four MT binding domains and lacks both 29-amino acid N-terminal acidic insert domains (generated by alternate splicing of exons 2 and 3). In addition, *UAS-tau^{AP}* has the following 14 point mutations: T111A, T153A, T175A, T181A, S199A, S202A, T205A, T212A, T217A, T231A, S235A, S396A, S404A, and S422A (based on numbering of the longest tau^{441} isoform). Transgenic lines of *UAS-tau^{WT}* and *UAS-tau^{AP}* were matched for equivalent levels of tau expression by quantitative Western blot for the studies shown in Figures 1–3 and 5. In Figure 4, a *UAS-tau^{AP}* line with a two-fold higher level of expression level as determined by quantitative Western blot analysis and

enzyme-linked immunosorbent assay (ELISA) was used. In addition, the following transgenic strains were used: *UAS-PKAmC* (Li et al., 1995); *UAS-hep* (Boutros et al., 1998); *UAS-cdk5-FLAG* (Connell-Crowley et al., 2000); *UAS-p35* (Ma and Haddad, 1999); and *UAS-wdb*^{DN} (Hannus et al., 2002). Transgene expression was achieved using the *GMR-GAL4* driver. Stocks and crosses were maintained on standard cornmeal-based *Drosophila* medium at 25°C.

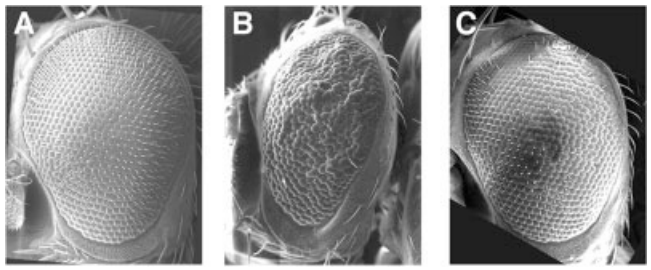

Fig. 3. Tau^{AP} is less toxic than tau^{WT} in the retina. **A:** Normal external eye of a control fly (*GMR-GAL4/+*), compared to the moderately rough eye produced by expression of tau^{WT} (**B**) as seen by scanning electron microscopy. Tau^{AP} shows no significant roughness (**C**). Transgenic lines are matched for equivalent levels of tau expression.

Western Blot and ELISA

Heads from adult flies at 1-day post-eclosion were homogenized in Laemmli buffer (Sigma, St. Louis, MO), boiled for 10 min at 100°C, and spun at 13,000× *g* for 2 min to remove insoluble debris. Proteins were resolved by SDS-polyacrylamide gel electrophoresis using a 10% separating gel (Cambrex, Rockland, ME), transferred to nitrocellulose (Bio-Rad, Hercules, CA), blocked in 5% milk in phosphate buffered saline with 0.1% Tween 20, and immunoblotted using one of the following antibodies: polyclonal anti-tau (AB1512; Chemicon, Temecula, CA) at 1:100,000 dilution; polyclonal anti-C terminal tau (Dako, Copenhagen, Denmark) at 1:10^6 dilution; AT8 (Innogenetics, Gent, Belgium) at 1:1000 dilution; Tau1 (Chemicon) at 1:10,000 dilution; AT180 (Innogenetics) at 1:1,000 dilution; TG3 (P. Davies) at 1:500 dilution; PHF1 (P. Davies) at 1:500 dilution. The appropriate anti-mouse or anti-rabbit HRP-conjugated secondary antibody was applied and signals were detected by chemiluminescence (Pierce, Rockford, IL). PKA activity was measured by ELISA, using a polyclonal antibody that recognizes tau phosphorylated at Serine 214 (pS214; Biosource, Camarillo, CA). The ELISA analysis represented in Figure 5A was carried out using flies at two-thirds of pupal life because this is the stage in which there is maximum AT100 (Endogen, Woburg, MA) immunoreactivity and degeneration. All other studies were carried out on adults. All data points indicated with asterisks are significant

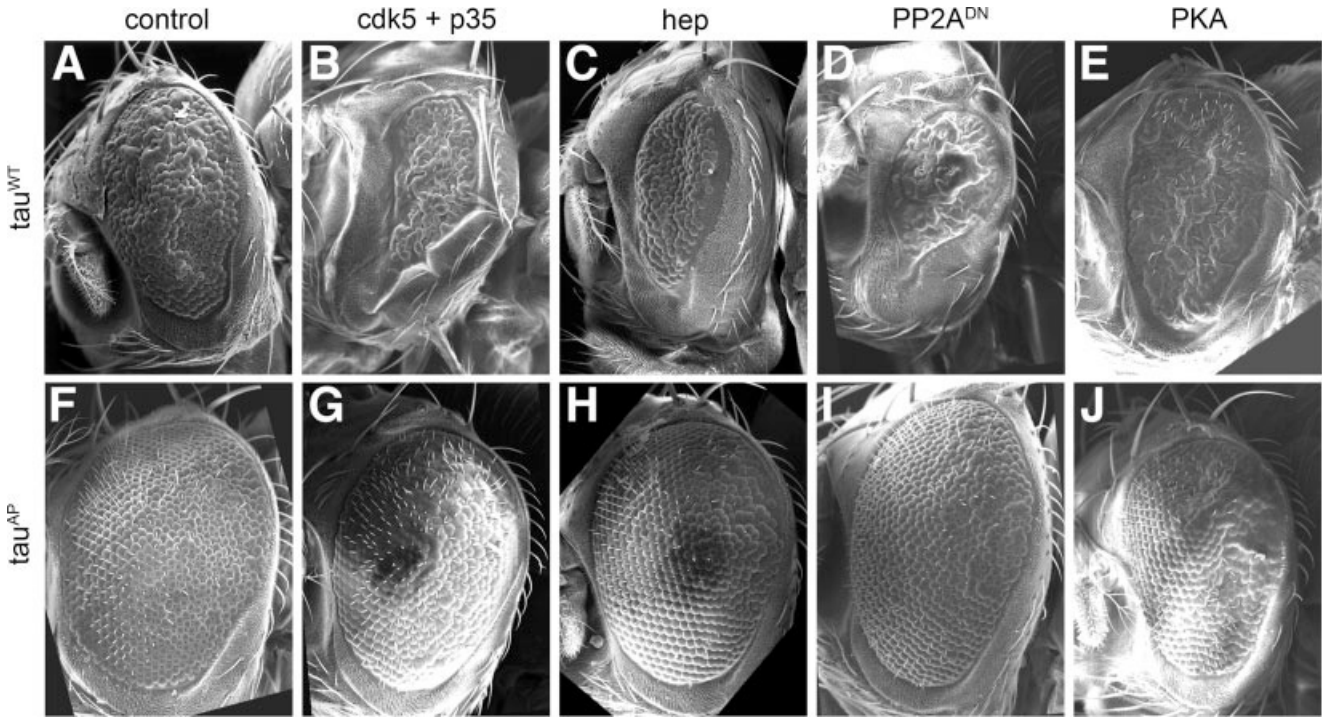

Fig. 4. Most kinase and phosphatase modifiers do not alter the toxicity of tau^{AP}. **A:** Scanning electron microscopy shows the moderate rough eye induced by expression of tau^{WT} in the retina compared to enhancement by cdk5 together with its activator p35 (**B**), the JNK kinase hemipterous (**C**), a dominant negative form of PP2A (**D**), and PKA (**E**). In contrast to the enhancement of tau^{WT}, the mild rough eye created by the expression of elevated levels of tau^{AP} (**F**) is not enhanced clearly by expression of cdk5 and its activator p35 (**G**), the JNK kinase hemipterous (**H**), or a dominant negative form of PP2A (**I**). However, the non-proline-dependent protein kinase PKA does enhance tau^{AP} retinal toxicity (**J**).

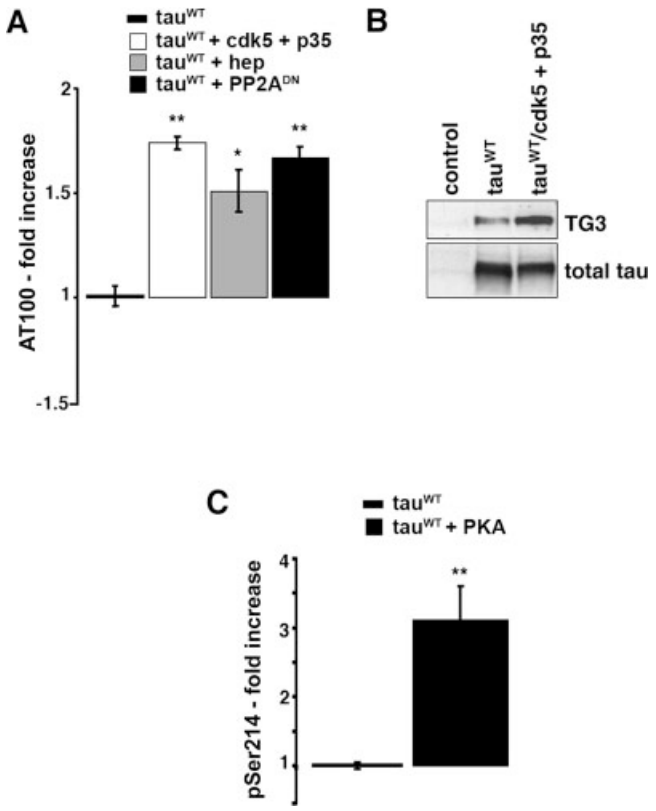

Fig. 5. Increased tau phosphorylation at disease-associated sites. **A:** Expression of cdk5 and its activator p35, hemipterous (hep), and a dominant negative form of PP2A (wdb^{DN}) all increase AT100 phosphorylation of tau^{WT} significantly as determined by ELISA. **B:** Expression of cdk5 and its activator p35 increases TG3 phosphorylation of tau^{WT} as determined by Western blot analysis. Control: *GMR-GAL4/+*. **C:** Expression of PKA significantly increases tau phosphorylation at Ser214 as determined by ELISA (using the pS214 polyclonal antibody from Biosource). Data points indicated with asterisks are significant. $\star P < 0.05$ or $\star\star P < 0.01$ (ANOVA with supplementary Student-Newman-Keuls test) for AT100 ELISA. $\star\star P < 0.01$, unpaired *t*-test for pSer214.

for ELISA ($\star P < 0.05$ or $\star\star P < 0.01$; ANOVA with supplementary Student-Newman-Keuls test).

Histology

Heads from adult flies at 1-day post-eclosion were fixed in formalin, embedded in paraffin, and 4-μm frontal sections prepared. Serial sections were cut through the entire head and placed on a single glass slide. Bielschowsky silver staining was carried out using standard protocols. Immunostaining on paraffin sections was carried out using either the avidin–biotin–peroxidase method or immunofluorescence. Antibodies recognizing Alz50, MCI, and TG3 were used at a dilution of 1:100. For the avidin–biotin–peroxidase slides, counterstaining with hematoxylin was carried out to highlight cortical nuclei.

Scanning Electron Microscopy

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.

Phosphatase Treatment

Homogenates from the heads of flies expressing either tau^{WT} or tau^{AP} were prepared in 1× lambda phosphatase buffer (New England Biolabs, Beverly, MA) containing a protease inhibitor cocktail (Roche, Indianapolis, IN) and incubated with 800 U of lambda protein phosphatase for 3 hr at 37°C. Homogenates were then subjected to SDS-PAGE and immunoblot analysis as described above.

RESULTS

Characterization of Tau^{AP}

To determine whether inhibiting phosphorylation of tau at major disease-associated sites could modify neurotoxicity, we generated a human tau construct (tau^{AP}) for expression in *Drosophila* in which all 14 of the Ser/Pro (SP) and Thr/Pro (TP) sites were mutated to alanine (Fig. 1A). Western blotting using phosphorylation-specific antibodies showed that endogenous kinases did not phosphorylate tau^{AP} at sites that have been mutated to alanine. As shown in Figure 1B, the phospho-specific antibodies AT8, AT180, TG3, and PHF1 recognized tau^{WT} but not tau^{AP}. Both tau^{WT} and tau^{AP} were recognized by a polyclonal antibody for total tau as well as by the Tau1 antibody, which preferentially recognizes unphosphorylated tau between amino acids 189–207.

Tau^{AP} migrated at a lower apparent molecular weight compared to tau^{WT} (Fig. 1B) suggesting that phosphorylation at SP/TP sites retarded the electrophoretic mobility of tau. To further investigate the phosphorylation state of tau, we treated both tau^{WT} and tau^{AP} with lambda protein phosphatase. Phosphatase treatment decreased the apparent molecular weight of both tau^{WT} and tau^{AP}, consistent with their phosphorylation by endogenous kinases in flies (Fig. 1C).

In addition to phosphorylation-specific epitopes, phosphorylation-independent conformations of tau have also been identified, which show relative specificity for disease states. The conformation-specific monoclonal antibodies MC1 and Alz50 recognize abnormal conformations of tau associated with AD and related disorders when used on tissue sections (Carmel et al., 1996; Vincent et al., 1996). Immunostaining analysis with a polyclonal antibody recognizing total tau showed the presence of equivalent levels and similar subcellular distribution of tau protein in the retinas of tau^{WT} and tau^{AP} flies (Fig. 2A–C). Although specific MC1 immunoreactivity was detected by immunofluorescence in the retinas of tau^{WT}-expressing flies (Fig. 2D,E) no MC1 signal was detected under the same conditions in retinas from flies expressing tau^{AP} (Fig. 2F). Using a higher sensitivity immunoperoxidase method, weak immunoreactivity for both MC1 and Alz50 was detected in tau^{AP} retinas (Fig. 2I,L). Phosphorylation of tau at SP/TP sites promotes the formation of disease-associated conformational states

of tau, but is not absolutely required for the formation of these epitopes.

Tau^{AP} Has Substantially Reduced Toxicity

To determine if blocking SP/TP phosphorylation altered tau neurotoxicity we compared the toxicity of tau^{WT} and tau^{AP} in the nervous system. We assessed toxicity by examining the retinas of flies expressing tau^{WT} or tau^{AP} (driver: *GMR-GAL4*). Wild-type fly eyes possess an ordered ommatidial morphology characterized by a regular array of lenses (Fig. 3A). Expression of tau^{WT} in the retina caused a moderate rough eye phenotype characterized by disorder of the ommatidial array and a modest decrease in the size of the eye (Fig. 3B). In contrast, expression of equivalent levels of tau^{AP} caused no toxicity as seen by the normal external morphology of the eye (Fig. 3C). Thus tau^{AP} was significantly less toxic than tau^{WT}. It should be noted that at higher levels, some toxicity is observed with tau^{AP} expression (Fig. 4F).

Kinase and Phosphatase Modifiers Target SP/TP Sites

Because the SP and TP target sites of proline-dependent protein kinases are mutated to alanine, tau^{AP} cannot be phosphorylated at SP/TP sites. We can thus use the tau^{AP} transgenic flies to determine if kinase and phosphatase modifiers act via SP/TP sites. We reported previously that manipulating the expression of cdk5 together with its activator p35, the JNK kinase hemipterous, PKA, and a dominant negative form of protein phosphatase 2A (PP2A, *widerborst*) modified the rough eye created by expression of the FTDP-17-linked mutant tau^{V337M} in the retina. Expression of each of these modifiers in the retina in the absence of transgenic human tau (*GMR-GAL4* driver) did not significantly affect eye morphology (Shulman and Feany, 2003). We first confirmed that these modifiers also modified the toxicity of tau^{WT}. Compared to the moderate rough eye created by expressing tau^{WT} in the retina (Fig. 4A), expression of cdk5 together with p35 (Fig. 4B), hemipterous (Fig. 4C), a dominant negative form of PP2A (Fig. 4D), and murine PKA (Fig. 4E) all enhanced the toxicity of tau^{WT}.

If cdk5/p35, hemipterous, and PP2A^{DN} enhanced the toxicity of tau^{WT} by increasing phosphorylation at SP/TP sites, then preventing phosphorylation by mutating these sites to alanine should block the observed enhancement. To test the ability of modifiers to enhance the retinal toxicity of tau^{AP}, we used a transgenic line that has a two-fold higher level of expression compared to tau^{WT}. Increasing the levels of tau^{AP} produced mild retinal toxicity with slight disruption of the normal ommatidial arrangement (Fig. 4F). The higher expressing tau^{AP} transgenic line was used to provide a more sensitive substrate for genetic modification than the lower expressing tau^{AP} line, which had no detectable phenotype in the retina (Fig. 3C). Expression of cdk5/p35, hemipterous, or PP2A^{DN} had no effect on the mild rough eye phenotype of tau^{AP} (Fig. 4G–I), consistent with the modifiers acting through SP/TP sites, most likely through direct phosphorylation of tau. In contrast, PKA, which phosphorylates tau at the non-SP site Ser214 *in vitro*, enhanced the toxicity of tau^{AP} (Fig. 4J).

Toxicity Correlates With Increased Phosphorylation at Disease-Associated Sites

To further test the relationship of SP/TP-site phosphorylation and tau toxicity we determined if co-expression of kinase and phosphatase modifiers with tau^{WT} influenced the phosphorylation state of tau. Sequential phosphorylation of tau at Thr212/Ser214 produces the AT100 epitope, which is a strongly disease-associated phospho-epitope (Zheng-Fischhofer et al., 1998). We used ELISA analysis to assess the level of phosphorylation at AT100 and found that co-expression of tau^{WT} with cdk5/p35, hemipterous, and PP2A^{DN} caused a statistically significant increase in phosphorylation (Fig. 5A). Similarly, expression of cdk5/p35 increased the level of phosphorylation at the TG3 site (Fig. 5B). ELISA analysis also showed a significant increase in phosphorylation at Ser214 in the presence of PKA (Fig. 5C). Together with our genetic results these studies support a direct effect of kinases and PP2A on tau phosphorylation.

DISCUSSION

Because mutations in the tau locus cause familial neurodegeneration with neurofibrillary tangle formation (Hong et al., 1998; Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998), primary abnormalities in tau can clearly induce neurodegeneration. However, the mechanisms leading to tau-mediated neuronal cell death still require further definition. In AD and related tauopathies, phosphorylation of tau is an early event that precedes the formation of neurofibrillary tangles and tangles themselves are rich in hyperphosphorylated tau, closely linking tau phosphorylation and neurodegeneration (Buee et al., 2000; Lee et al., 2001). Not surprisingly, therefore, the kinases and phosphatases that control tau phosphorylation have been studied extensively. Both in vivo and in vitro data suggest that several kinases (cdc2, cdk2, cdc5, MAPK (ERK1/2), GSK3, PKA, JNK) and the phosphatase PP2A are the main regulators of phosphorylation at SP/TP sites (Gong et al., 2005).

To assess the relationship between phosphorylation and neurotoxicity in vivo, tau kinases and phosphatases including GSK3 (Spittaels et al., 2000; Lucas et al., 2001; Jackson et al., 2002), the cdk5 activator p25 (Ahlijanian et al., 2000; Bian et al., 2002; Cruz et al., 2003; Noble et al., 2003), and a dominant negative form of PP2A (Kins et al., 2001), have been overexpressed in animal models. In all of these models, overexpression of GSK3, p25, or mutant PP2A caused an increase in tau phosphorylation; however, the effects on neurodegenerative cell death were variable. For example, when p25 was overexpressed alone (Ahlijanian et al., 2000; Cruz et al., 2003) or together with human tau^{P301L} (Noble

et al., 2003) there was significant tau aggregation and neurofibrillary pathology. In conditions of marked p25 overexpression, there was also progressive neuronal loss (Cruz et al., 2003). When Bian et al. (2002) overexpressed human p25, however, they observed axonal degeneration and tau hyperphosphorylation in the absence of neurofibrillary tangle formation. Similarly, Lucas et al. (2001) and Jackson et al. (2002) found that overexpression of GSK3 resulted in neurotoxicity, whereas Spittaels et al. (2000) found that axonal pathology was actually decreased, suggesting that GSK3 may positively or negatively regulate neurodegeneration regardless of tau hyperphosphorylation in these models.

In addition to altering kinase expression genetically, the pharmacologic inhibition of GSK3 and other kinases decreased significantly tau phosphorylation, levels of aggregated insoluble tau, and neuropathology in vivo (Noble et al., 2005; Le Corre et al., 2006). Our work and the studies by others (Shulman and Feany, 2003; Khurana et al., 2006), support the notion that altering kinase and phosphatase expression can influence tau neurotoxicity in vivo; however, the direct target of kinases, phosphatases, and drug treatments has often remained unclear.

Having first established that tau^{WT} was phosphorylated effectively by endogenous kinases in *Drosophila* (Fig. 1), we addressed whether tau phosphorylation contributed directly to neurodegeneration in vivo. We expressed a mutant form of tau (tau^{AP}) in which the disease-associated SP/TP sites cannot be phosphorylated. In fly head homogenates, tau^{AP} was not recognized by the phosphorylation-specific antibodies AT8, AT180, TG3, or PHF1 (Fig. 1), showing that these sites were not phosphorylated in vivo. Importantly, when expressed at levels similar to tau^{WT}, tau^{AP} showed no evidence of toxicity in the retina (Fig. 3). These results show that SP/TP phosphorylation sites are critical for tau neurotoxicity in vivo.

We also noted that tau^{AP} migrated more rapidly than tau^{WT} on SDS-PAGE (Fig. 1). Because phosphorylation at SP/TP sites is known to comprise >80% of total tau phosphorylation (Biernat and Mandelkow, 1999), it was not surprising that blocking phosphorylation at the SP/TP sites accelerated the mobility of tau^{AP}. Although not phosphorylated at SP/TP sites, phosphatase treatment suggested that tau^{AP} was phosphorylated at other sites (Fig. 1C), and thus SP/TP-independent phosphorylation may be responsible for the residual toxicity we observed when high levels of tau^{AP} were expressed in the retina (Fig. 4).

To test whether specific kinases and phosphatases enhance tau toxicity via SP/TP sites, we analyzed three tau modifiers that are good candidates for directly modulating tau phosphorylation at SP/TP sites: the proline-directed protein kinase cdk5 and its activator p35 (cdk5/p35), the JNK kinase hemipterous, and a dominant negative mutant form of PP2A. Each of these modifiers has been implicated in human disease and we showed previously that they all enhance the rough eye phenotype of the FTDP-17 linked mutant tau^{V337M} in *Drosophila* (Shulman and Feany, 2003). Co-expression of tau^{WT} with cdk5/p35, hemipterous, or $PP2A^{DN}$ significantly increased tau toxicity (Fig. 4). When the SP/TP sites were mutated to alanine, however, tau toxicity was no longer enhanced by cdk5/p35, hemipterous, and $PP2A^{DN}$ (Fig. 4), showing that these modifiers require intact SP/TP phosphorylation sites. We also showed that enhancement of tau toxicity by these modifiers was associated with an increase in phosphorylation at SP/TP sites. ELISA analysis showed that cdk5/p35, hemipterous, and $PP2A^{DN}$ increased phosphorylation significantly at AT100. In addition, co-expression of cdk5/p35 with tau^{WT} increased phosphorylation at TG3 (Fig. 5). These results suggest strongly that cdk5/p35, hemipterous, and $PP2A^{DN}$ enhance tau toxicity by affecting phosphorylation directly at SP/TP sites.

If the residual toxicity of tau^{AP} (Fig. 4) is indeed controlled through SP/TP-independent phosphorylation, then a kinase that targets non-SP/TP sites might enhance tau^{AP} toxicity. PKA phosphorylates tau at several sites, including serines within the microtubule binding domains and Ser214 in the proline rich region (Zheng-Fischhofer et al., 1998; Jicha et al., 1999b; Schneider et al., 1999). Because PKA enhanced the eye phenotype of tau^{AP}, phosphorylation of tau^{AP} at Ser214 or other non-SP/TP sites may lead to toxicity. Thus, tau^{AP} can be used to determine whether modifiers work through SP/TP sites or via other mechanisms.

Phosphorylation of tau by certain kinases, such as PKA and MARK, promotes its detachment from microtubules (Schneider et al., 1999), which was shown to facilitate microtubule-based axonal trafficking (Mandelkow et al., 2004). In addition, direct binding of tau to microtubules was found to reduce the attachment of kinesin motors (Seitz et al., 2002), which may account for the ability of tau to interfere with microtubule-associated transport of vesicles and organelles in cultured cells (Ebneth et al., 1998; Stamer et al., 2002). Interestingly, our finding that PKA enhances tau^{AP}-induced degeneration, suggests that detachment of tau from microtubules may increase toxicity in our system.

In addition to phosphorylation-specific tau epitopes, several conformationally specific epitopes have also been correlated with disease (Carmel et al., 1996; Jicha et al., 1997). In AD, tau is recognized not only by phosphorylation-specific antibodies, but also conformation-specific antibodies such as Alz50 and MC1. Specifically, Alz50 and MC1 recognize unique intramolecular conformations of tau that include amino acids at the extreme amino terminus in addition to amino acids 312–322 located in the third microtubule binding domain (Jicha et al., 1997, 1999a). Phosphorylation is believed to contribute to high affinity binding of Alz50 and MC1 by stabilizing disease-associated conformations of tau (Carmel et al., 1996). Interestingly, immunoreactivity with Alz50 and MC1 precedes the appearance of filamentous aggregates in AD (Hyman et al., 1988; Carmel et al., 1996; Jicha et al., 1999a). We found that SP/

TP phosphorylation promoted abnormal tau conformations in *Drosophila* as well because retinal tissue from flies expressing tau^{WT} was strongly immunoreactive for Alz50 and MC1 whereas tau^{AP}-expressing flies were only weakly immunoreactive (Fig. 2). Thus, although phosphorylation of the SP/TP sites is not required for tau to adopt altered conformations found in disease states, it strongly promotes these conformations in vivo.

Our results, together with previous work in the field, suggest strongly that tau phosphorylation is critical for neurotoxicity in vivo. Blocking phosphorylation at SP/TP sites reduced toxicity of tau in the retina of transgenic flies markedly. Conversely, elevated phosphorylation at these sites correlated with increased toxicity. Furthermore, our phosphorylation-incompetent form of tau showed that modifiers predicted to target SP/TP motifs, including cdk5/p35, hemipterous, and $PP2A^{DN}$ require these sites to enhance tau toxicity in vivo, and most likely act on these sites directly. Our findings thus identify SP/TP sites as important mediators of tau toxicity in vivo and support targeting these sites in developing disease therapies.

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