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Article

TOR-Mediated Cell-Cycle Activation Causes Neurodegeneration in a *Drosophila* Tauopathy Model

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Summary

Background: Previous studies have demonstrated re-expression of cell-cycle markers within postmitotic neurons in neurodegenerative tauopathies, including Alzheimer's disease (AD). However, the critical questions of whether cell-cycle activation is causal or epiphenomenal to tau-induced neurodegeneration and which signaling pathways mediate cell-cycle activation in tauopathy remain unresolved.
Results: Cell-cycle activation accompanies wild-type and mutant tau-induced neurodegeneration in *Drosophila*, and genetically interfering with cell-cycle progression substantially reduces neurodegeneration. Our data support a role for cell-cycle activation downstream of tau phosphorylation, directly preceding apoptosis. We accordingly show that ectopic cell-cycle activation leads to apoptosis of postmitotic neurons in vivo. As in AD, TOR (target of rapamycin kinase) activity is increased in our model and is required for neurodegeneration. TOR activation enhances tau-induced neurodegeneration in a cell cycle-dependent manner and, when ectopically activated, drives cell-cycle activation and apoptosis in postmitotic neurons.
Conclusions: TOR-mediated cell-cycle activation causes neurodegeneration in a *Drosophila* tauopathy model, identifying TOR and the cell cycle as potential therapeutic targets in tauopathies and AD.

Introduction

AD and tauopathies are neurodegenerative diseases characterized by abnormal accumulations of hyperphosphorylated tau, a microtubule-associated protein [1]. While the discovery that dominant mutations in the *TAU* gene cause hereditary frontotemporal dementias has directly implicated tau in disease pathogenesis [2], the mechanisms through which tau drives neurodegeneration remain elusive. Intriguingly, several studies have described aberrant neuronal expression and localization of cell-cycle proteins in postmortem tissue from patients with tauopathies and AD [3]. Several positive regulators of the G1/S and G2/M cell-cycle transitions are aberrantly expressed or localized, including cyclins and cyclin-dependent kinases (Cdks) [4], the S-phase marker proliferating cell nuclear antigen (PCNA [5]), and the M-phase marker phosphohistone-3 (PH3 [6]). In addition, one study has demonstrated that neurons in AD replicate their DNA prior to dying [7]. While these findings raise the possibility that cell-cycle activation is deleterious for postmitotic neurons, two critical issues remain unresolved. First, it has been unclear whether there is a causal relationship in vivo between cell-cycle activation and neurodegeneration [8]. The upregulation of negative cell-cycle regulators (including p27kip/waf) in AD has further complicated the functional relationship between cell-cycle activation and neurodegeneration [9]. Second, the signaling pathways mediating cell-cycle activation in neurodegenerative tauopathies have not been determined. Neuronal markers for several mitogenic signaling pathways are aberrantly upregulated in AD, including the target of rapamycin (TOR) kinase pathway [10–12], a downstream effector of insulin signaling known to regulate growth and lifespan [13, 14]. However, the significance of these pathways in disease has not been determined, nor whether their activation can lead to reactivation of cell cycle in postmitotic neurons. Indeed, inconsistencies in the reported effects of these pathways on neuronal survival in cell-culture systems [15, 16] underscore the need for investigation in an animal model [8].

Genetic analysis in *Drosophila* is well suited to address the issue of causality. The relationship among tau-induced neurodegeneration, cell-cycle activation, and mitogenic signaling pathways in vivo can appropriately be investigated in flies because, first, key features of human tauopathies, including tau hyperphosphorylation and progressive neurodegeneration, are recapitulated by transgenic expression of human or mutant wild-type tau [17–19]. Second, fly and mammalian cell-cycle machineries are substantially conserved, as are mitogenic signaling pathways including the TOR pathway [20]. In *Drosophila*, as in mammalian cells, Rheb activates TOR and drives cells through the G1/S cell-cycle transition, and Tsc proteins restrict tissue growth and reduce cell size and cell proliferation by directly inhibiting Rheb. Activation of TOR signaling leads to phosphorylation of S6k at Thr389 in mammalian cells, and a recent study has shown this epitope is elevated in AD tissue [10]. TOR activity can similarly be measured in *Drosophila* by detecting phosphorylation of S6k at Thr398, the corresponding site in the fly protein [20].

In this study we show that cell-cycle activation accompanies wild-type and mutant tau-induced apoptotic neurodegeneration in *Drosophila*. Genetic and pharmacologic inhibition at both the G1/S and G2/M cell-cycle transitions blocks neuronal apoptosis,

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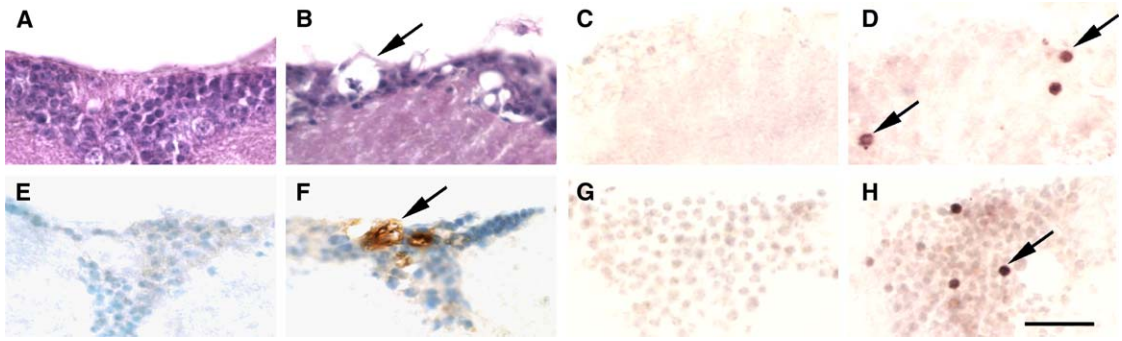

Figure 1. Cell-Cycle Activation Accompanies Neurodegeneration in 10-Day-Old Tau^{R406W} Transgenic Flies

(A and B) Hematoxylin and eosin-stained nontransgenic control fly brain demonstrating normal numbers and morphology of cortical neurons (A) compared to tau transgenic fly in which neuronal vacuolization and nuclear fragmentation is seen (B, arrow).

(C–H) TUNEL (C and D), PCNA (E and F), and PH3 (G and H)-positive neurons are absent in controls (C, E, G) but are prominent in tau transgenics (D, F, H), as indicated by arrows. A blue (hematoxylin) nuclear counterstain is used in (E) and (F). Scale bar equals 10 μm.

establishing a clear causal relationship between cell-cycle activation and tau-induced neurodegeneration. Our data implicate cell-cycle activation downstream of tau phosphorylation and directly preceding apoptosis. Finally, as in human disease, TOR activity is upregulated in our model. TOR is required for neurodegeneration and, furthermore, drives cell-cycle activation in postmitotic neurons and enhances tau-induced neurodegeneration in a cell cycle-dependent manner. Our results thus causally implicate cell-cycle activation in tau-induced neurodegeneration and identify TOR signaling as an important pathway through which tau activates the cell cycle.

Results

Cell-Cycle Activation Accompanies Neurodegeneration in a *Drosophila* Tauopathy Model

We first determined whether cell-cycle activation accompanied neurodegeneration in a fly model of tauopathy. We have previously reported that expression of a mutant form of tau linked to familial frontotemporal dementia, tau^{R406W}, in the fly brain (panneural driver: *ELAV-GAL4*) leads to progressive neurodegeneration [17]. At eclosion, the brains of tau-expressing flies appeared morphologically normal, but by 10 days clear neurodegeneration was observed, characterized histologically by condensation and fragmentation of neuronal nuclei and vacuolization (Figures 1A and 1B). TUNEL staining identified apoptotic neurons in tau transgenic animals but not in age-matched controls (Figures 1C and 1D).

We immunostained for PCNA and PH3 to assess early and late cell-cycle activation. Control animals were completely negative for PCNA and PH3 at 10 days (Figures 1E and 1G) and 30 days (data not shown). In contrast, brains from tau transgenic flies showed prominent expression of both PCNA (Figure 1F) and PH3 (Figure 1H) at 10 days. PCNA staining was particularly prominent in areas of neurodegeneration, as indicated by characteristic nuclear changes and cytoplasmic vacuolization (Figure 1F).

Together, these findings demonstrate that abnormal activation of the cell cycle accompanies tau-induced apoptotic neurodegeneration in *Drosophila* and suggest that cell-cycle activation is likely to be a relatively late event in our model.

Cell-Cycle Activation Mediates Mutant Tau-Induced Neurodegeneration

In flies, entry into the G1/S transition is coordinated by the transcription factor E2F1/DP and Cdk2 complexed to Cyclin E (Cdk2/Cyclin E; Figure 2A). Transgenic coexpression of the E2F1 inhibitor Retinoblastoma factor-1 (Rbf1) and the Cdk2 inhibitor Dacapo (Dap; the *Drosophila* homolog of human p21/p27) synergistically block the G1/S transition (Figure 2A; [21]). We coexpressed Dap and Rbf1 with tau in the fly brain and found a significantly reduced number of TUNEL-positive cells in the brain compared to age-matched tau transgenic flies (Figure 2B). β-galactosidase (β-gal) was used to control for nonspecific effects of expressing tau together with an additional transgene.

In *Drosophila,* Cdk1 (cdc2) and Cyclin A form a complex (Cdk1/Cyclin A) that catalyzes the G2/M transition, and Cdk1/Cyclin B is required for progression through mitosis (Figure 2A). We found that the number of TUNEL-positive cells was significantly reduced by coexpressing tau with Dap and Cdk1^{E51Q}, a dominant-negative form of Cdk1 [22] (Cdk1^{DN}; Figure 2B), and significantly increased by coexpressing Cyclin A with tau (Figure 2C). Cyclin A did not produce significant neurodegeneration when expressed alone (Figure 2C). Coexpression of Cyclin E or Cdk1/Cyclin B in the brain with tau was lethal or semilethal, consistent with enhanced toxicity. Coexpressing Cdk4/Cyclin D, which drives growth and cell cycle in flies, was semilethal. Immunoblotting demonstrated equivalent tau protein levels in 10-day-old tau-expressing flies and flies coexpressing tau with cell-cycle modifiers (not shown), indicating that the genetic modification observed was not attributable to altered tau expression. Expressing cell-cycle modifiers in the brain without tau did not change gross brain morphology or cellularity (not shown).

Cell-cycle modification of neurodegeneration was apparent on routine histological analysis. In control flies, the neuronal projections in the lamina appear as regular longitudinal bundles in frontal sections (Figure 2E). The lamina of 1-day-old flies expressing tau (Figure 2G), tau together with Dap and Rbf1 (Figure 2I), or tau with

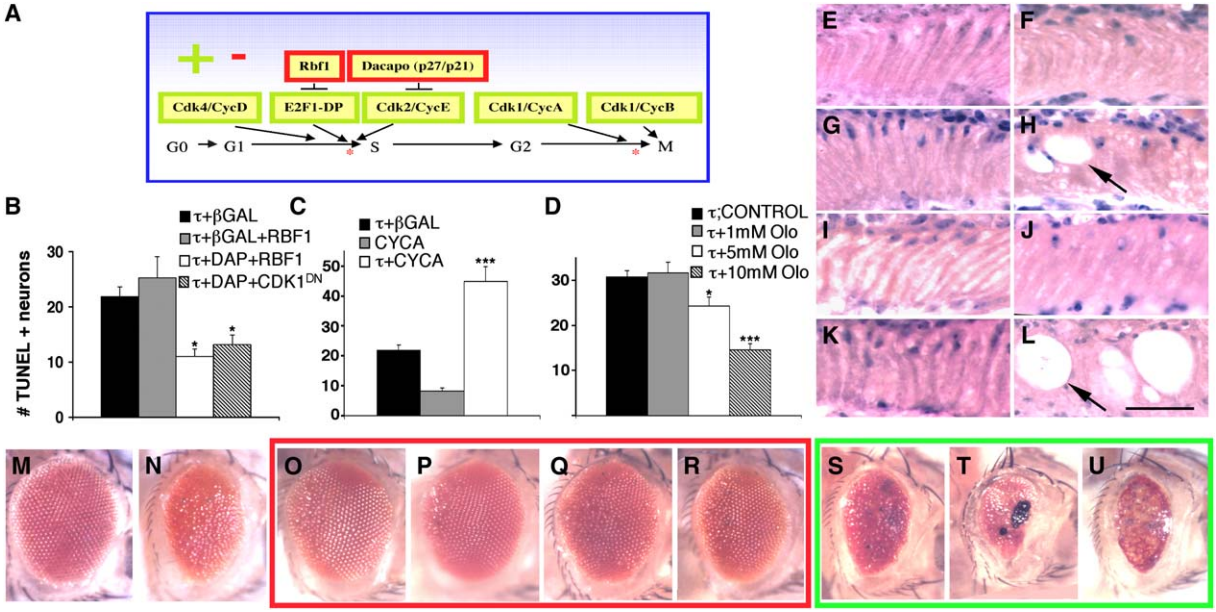

Figure 2. Modulation of the Cell Cycle Modifies Neurodegeneration in the Brain and Retina of Transgenic Tau Flies

(A) The cell-cycle machinery is highly conserved between *Drosophila* and mammalian cells.

(B–D) Genetic and pharmacologic cell-cycle modulation modifies neurodegeneration in transgenic tau^{R406W} fly brains. Neurodegeneration is suppressed by coexpressing Dap and Rbf1 or Dap and $Cdk1^{DN}$ (B) and enhanced by Cyclin A (C). Neurodegeneration is suppressed by feeding the Cdk inhibitor drug olomoucine (D). Statistically significant differences from control groups are indicated (one-way ANOVA with Student-Neuman-Keuls; *$p < 0.05$; ***$p < 0.001$). Error bars: ± SEM.

(E–L) Cell-cycle modulation modifies tau-mediated degeneration in the lamina. Lamina structure is normal in control flies at 1 (E) and 10 (F) days and in 1-day-old flies expressing tau^{R406W} with β-gal (G), tau^{R406W} with Dap and Rbf1 (I), or tau^{R406W} with Cyclin A (K). Vacuolar degeneration in flies coexpressing tau^{R406W} with β-gal at 10 days (H, arrow) is suppressed by coexpressing tau^{R406W} with Dap and Rbf1 (J) and enhanced by coexpressing tau^{R406W} with Cyclin A (L, arrow). Scale bar equals 10 μm.

(M–U) Cell-cycle modulation modifies the mutant tau^{V337M}-induced rough-eye phenotype. Compare normal eye structure (M) to the tau^{V337M}-induced rough eye (N). The tau^{V337M}-induced rough eye is strongly suppressed by coexpressing tau^{V337M} with Dap and Rbf1 (O), Dap and $Cdk1^{DN}$ (P), or by removing one copy of *E2f1* (Q) or *Cyclin A* (R). The tau^{V337M}-induced retinal pathology is enhanced by coexpressing tau^{V337M} with Cdk4/Cyclin D (S), Cyclin B (T), or Cyclin A (U).

Cyclin A (Figure 2K) exhibited regular organization. While the lamina of 10-day-old control flies appeared normal (Figure 2F), vacuolar degeneration of the lamina occurred in tau flies aged to 10 days (Figure 2H). This degeneration was rescued by coexpression of Dap and Rbf1 (Figure 2J) and was strikingly enhanced by Cyclin A (Figure 2L). Flies expressing Cyclin A alone did not exhibit vacuolization (not shown).

These genetic data implied that cell-cycle activation at both the G1/S and G2/M transitions contributes to tau-induced neurodegeneration in vivo. We also found that pharmacologic inhibition of the cell cycle ameliorated tau-induced neurodegeneration, because feeding tau-expressing flies olomoucine reduced the number of TUNEL-positive neurons significantly by 10 days (Figure 2D). Olomoucine is a relatively specific pharmacologic Cdk inhibitor developed as a potential cancer therapy [23] and previously shown to block cell cycle-dependent apoptosis in cultured neurons [24].

Expressing tau in the retina (driver: *GMR-GAL4*), including two forms of mutant tau (tau^{V337M} and tau^{R406W}), results in degeneration of photoreceptor neurons and gives a rough eye [17–19]. This phenotype consists of a reduction in eye size and loss of regular ommatidial arrangement (compare Figures 2M and 2N). We utilized this phenotype to further examine genetic interactions between tau-induced neurodegeneration and cell-cycle components. From the two mutant forms of tau, we selected a tau^{V337M} line [25] that had a moderate rough eye and was therefore a suitable substrate for genetic modification.

As with panneural expression (Figure 2B), coexpressing βgal and Rbf1 with mutant tau did not substantially ameliorate retinal toxicity (see Figure S1C in the Supplemental Data available with this article online). However, coexpressing both Dap and Rbf1 (Figure 2O) or human p21 and Rbf1 (not shown) significantly suppressed the rough eye. Rescue was also achieved by coexpressing Dap and $Cdk1^{DN}$ (Figure 2P) or Rbf1 and $Cdk1^{DN}$ (not shown). A null allele, $E2F1^{91}$ (Figure 2Q), and two deficiencies that uncover the *E2F1* locus (not shown) dominantly suppressed the phenotype, as did loss-of-function alleles of Cyclin B3 ($CycB3^{L6540}$; Figure S1D) and Cyclin A ($CycA^{C8LR1}$; Figure 2R). Expression of Cdk4/Cyclin D (Figure 2S), Cyclin B (Figure 2T), Cyclin A (Figure 2U), Cyclin E (Figure S1F), Cdk1/Cyclin B (Figure S1H), and Cyclin B3 (not shown) markedly enhanced the rough eye. Expression of these positive cell-cycle regulators alone did not significantly alter the structure of the eye (Figures S1E and S1G; not shown). Coexpression of Cdk1 or Cdk2 without their cyclin partners had no effect (not shown). These data substantiate and extend our finding in the brain that mutant tau-induced neurodegeneration is cell-cycle dependent.

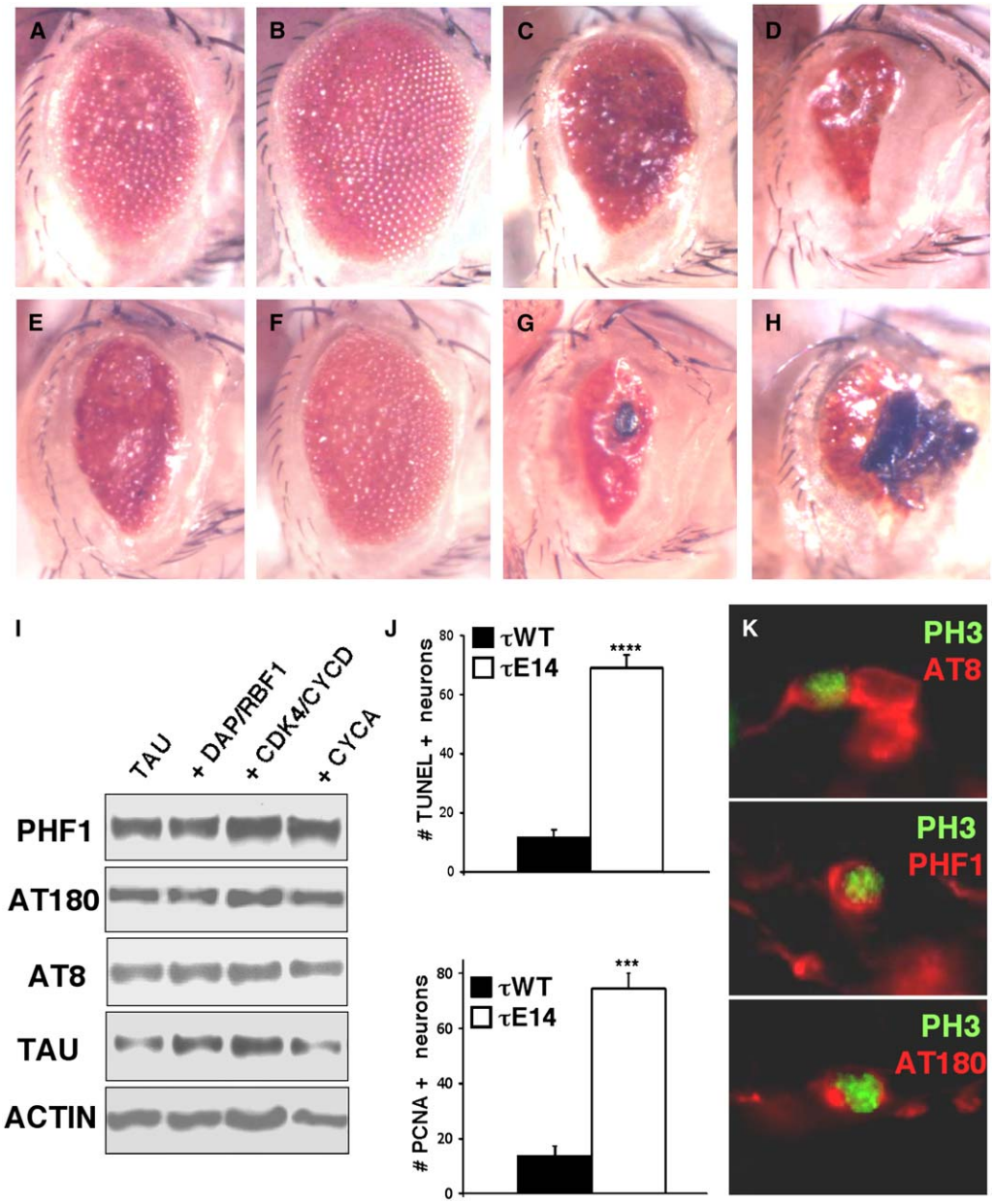

Figure 3. Cell-Cycle Activation Is Downstream of Tau Phosphorylation

(A–D) Cell-cycle modulation modifies the tau^{WT}-induced rough-eye phenotype. The tau^{WT}-induced rough eye (A) is suppressed by coexpression of Dap and Rbf1 (B) and enhanced by coexpression of either Cdk4/Cyclin D (C) or Cyclin A (D).

(E–H) Cell-cycle modulation modifies the pseudophosphorylated tau^{E14}-induced rough-eye phenotype in which all Ser/Pro and Thr/Pro kinase target sites are mutated to glutamate. The tau^{E14}-induced rough eye (E) is suppressed by coexpression of Dap and Rbf1 (F) and enhanced by coexpression of Cdk4/Cyclin D (G) or Cyclin E (H).

(I) Cell-cycle modifiers depicted in (A)–(D) do not alter levels of phosphoepitopes PHF1 (S396, S404), AT180 (T231, S235), or AT8 (S202, T205). Blots were each reprobed with a phospho-independent antibody to tau (TAU) and actin (ACTIN), shown here for the AT8 experiment. Quantitation from $\geq$3 independent experiments indicated no statistically significant differences (one-way ANOVA with post hoc for multiple comparisons).

(J) At equivalent expression levels to tau^{WT}, tau^{E14} induces significantly more apoptotic neurodegeneration in the brain accompanied by substantially higher numbers of PCNA-positive neurons (unpaired t test; ***$p < 0.001$; ****$p < 0.0001$; error bars: ± SEM). Flies aged to 30 days.

(K) PH3 (green; nuclear stain) frequently colocalizes with AT8, PHF-1, and AT-180 (red; cytoplasmic stain) in the brains of mutant tau^{R406W}-expressing flies. All flies shown at 10 days.

Cell-Cycle Modulation Modifies Wild-Type Tau-Induced Neurodegeneration

AD and sporadic tauopathies are associated with abnormal neuronal accumulation of wild-type tau (tau^{WT}). Expressing tau^{WT} is less toxic to neurons than expressing mutant tau [17], but also induces progressive neurodegeneration in flies that is PCNA positive at 30 days (Figure 3J). Retinal expression of tau^{WT} gives a rough eye (Figure 3A; [18]), and we found that cell-cycle modulators (Figures 3B–3D) modified this phenotype as effectively as for mutant tau, indicating that tau^{WT}-induced neurotoxicity is also cell-cycle dependent.

Cell-Cycle Modulation Modifies Tau Toxicity without Altering Phosphorylation at Key Serine and Threonine Sites

Multiple lines of evidence implicate tau hyperphosphorylation in neurodegeneration [1]. Since many disease-associated phosphoepitopes are generated by phosphorylation at Ser-Pro and Thr-Pro motifs and Cdks are proline-directed kinases, we tested whether cell-cycle manipulations modified tau toxicity through altering tau phosphorylation.

Accumulation of abnormally hyperphosphorylated tau and the presence of disease-associated phosphoepitopes accompanies tau-induced neurodegeneration [17–19]. We focused on three disease-associated phosphoepitopes, AT8, PHF-1, and AT180, which can be created by Cdks in vitro and during mitosis of cultured cells [26]. We found that these epitopes accompanied retinal (Figure 3I) and brain (Figure 3K; [17]) degeneration in our tauopathy model. However, quantitation of at least three separate experiments revealed no statistically significant changes in any of these epitopes (AT8, $p > 0.5$; PHF-1, $p > 0.2$; AT180, $p > 0.8$; one-way ANOVA with Student-Neuman-Keuls post hoc) despite dramatic modification of toxicity (Figures 3A–3D and 2).

Tau-Induced Cell-Cycle Activation Depends upon Tau Phosphorylation

The inability of cell-cycle modulation to alter tau phosphoepitopes (Figure 3I) raised the possibility that cell-cycle activation might occur downstream of tau phosphorylation. To test this, we first created a pseudo-phosphorylated tau construct in which all 14 Ser-Pro and Thr-Pro kinase target sites were mutated to glutamate (tau^{E14}). We found that, when expressed at equivalent levels to tau^{WT}, tau^{E14} not only induced enhanced toxicity in the brain but also substantially more PCNA-positive (Figure 3J) and PH3-positive (not shown) neurons, consistent with cell-cycle activation being downstream of tau phosphorylation. To further support this model, tau^{E14}-induced retinal toxicity (Figure 3F) was clearly modified by cell-cycle manipulation (Figures 3E–3H), indicating that cell-cycle modification of tau-dependent neurotoxicity is not mediated through Ser-Pro/Thr-Pro phosphorylation sites.

To substantiate the dependence of cell-cycle activation upon tau phosphorylation, we expressed tau in the brain in a *shaggy* (*sgg*) loss-of-function genetic background. Sgg is the fly homolog of glycogen synthase kinase-3 (GSK-3), a Ser/Thr kinase and well-established modifier of tau toxicity and phosphorylation in flies [18]. Overexpressing *sgg* has previously been shown to increase generation of the AT100 tau phosphoepitope [18, 19], and we found that AT100 levels were reduced in a *sgg* mutant background (Figure S2A). Importantly, this reduction was accompanied by a significantly fewer PCNA-positive neurons (Figure S2B). These data, together with the tau^{E14} analysis (Figure 3J), indicate that altering tau phosphorylation modulates cell-cycle activation, while cell-cycle modifiers do not change tau phosphorylation (Figure 3I).

Finally, we observed frequent colocalization between PH3 and tau phosphoepitopes in tau^{R406W} transgenic flies (Figure 3K; driver: *ELAV-GAL4*) and used immunohistochemical methods to quantify this. In the central body complex, we quantified the number of PH3-positive cells (mean = 107.5/hemibrain; $n \geq 6$ hemibrains) that were also tau phosphoepitope positive. We found that the majority of PH3-positive neurons (>90%) were also immunoreactive for AT8, PHF-1, or AT180, consistent with tau phosphorylation preceding cell-cycle activation. In contrast, we found that approximately 90%, 50%, and 20% of neurons were immunoreactive for AT8, PHF-1, and AT180, respectively, in a well-defined area of the cortex, the caylx of the mushroom body ($n = 8$ hemibrains). These numbers were representative of overall prevalence throughout the brain. Thus, the significant overlap of PH3 and disease-related phosphoepitopes did not occur by chance alone. Our biochemical, genetic, and immunohistochemical data strongly suggest that cell-cycle activation is dependent upon and downstream of tau phosphorylation in our model.

Ectopic Cell-Cycle Activation Leads to Apoptotic Neurodegeneration in the Adult Fly Brain

If cell cycle directly mediates apoptosis in our tau model, then ectopic cell-cycle activation should lead to apoptotic neurodegeneration in the absence of transgenic tau. We activated the cell cycle by ectopically expressing positive regulators of the G1/S transition (Figure 2A). Expressing E2F1/DP in the brain with the *ELAV-GAL4* driver is lethal. We therefore selectively induced E2F1/DP expression in the adult fly brain (driver: *ELAV-GeneSwitch* [27]). At 10 days, there was marked loss of cortical neurons and rarefaction of the underlying neuropil compared to controls (Figures 4A and 4B). Many neurons were strongly immunolabeled with anti-PCNA (Figure 4C) and anti-PH3 (Figure 4D) and were TUNEL positive (Figure 4E). These markers were also observed in the brains of Cyclin E-expressing flies (Figures 4F–4H), while nontransgenic control flies were negative for these markers (Figure 1; data not shown).

Thus, cell-cycle activation in postmitotic neurons leads to neuronal apoptosis in vivo. The converse, however, is not true because direct induction of the proapoptotic gene *reaper* in adult neurons did not lead to cell-cycle activation, despite dramatic induction of apoptosis (Figures 4I and 4J).

The TOR Pathway Mediates Cell-Cycle Activation and Tau-Induced Neurodegeneration

TOR activation drives growth and cell-cycle progression in *Drosophila* [28, 29]. To investigate TOR signaling as a possible upstream event in tau-mediated cell-cycle activation, we first determined whether TOR activity was increased in our model. We used an antibody specific for the activated form *Drosophila* S6k, phosphorylated at Thr398 (P-S6k). We found that expressing tau in the brain induced a marked increase in P-S6k (Figure 5B). Interestingly, total S6k levels were decreased in the brain when S6k activity was increased by either tau or Rheb expression (Figure S4).

To determine whether blocking TOR activity pharmacologically or genetically could reduce mutant tau-induced neurodegeneration in the brain, we first fed flies rapamycin, a macrolide antibiotic that is a specific TOR inhibitor [13]. Rapamycin treatment reduced the number of TUNEL-positive cells (Figure 5C) and neurodegenerative vacuoles (not shown) by 10 days. We

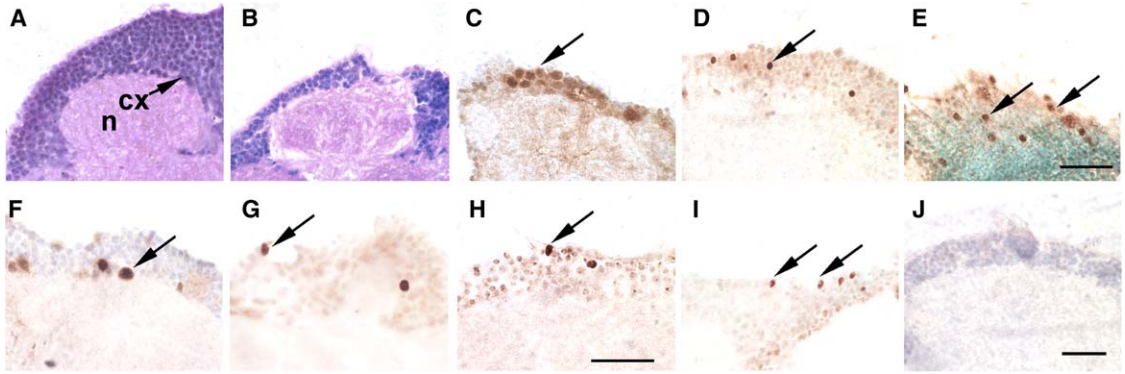

Figure 4. Ectopic Cell-Cycle Activation Leads to Degeneration of Adult Postmitotic Neurons In Vivo

(A–E) Expression of E2F1-DP in the brain induces cell-cycle activation and neurodegeneration. The normal appearance of mushroom body cortex (cx) and neuropil (n) in control fly is lost after expression of E2F1-DP, which induces cortical neuron loss and neuropil rarefaction (B). Neurons are immunopositive for PCNA (C, arrow) and PH3 (D, arrow) and are TUNEL positive (E, arrows). All flies are aged to 10 days. Hematoxylin and eosin staining is used in (A) and (B). Nuclear counterstain is used in (C) (hematoxylin; blue) and (E) (methyl green; green). Scale bar equals 10 μm.

(F–H) Expression of Cyclin E in the brain induces cell-cycle activation and neurodegeneration. Cyclin E induces PCNA expression (F, arrow), PH3 expression (G, arrow), and positive TUNEL staining (H, arrows) in neurons. Flies are aged to 10 days. Nuclear counterstain is used in (F) (hematoxylin; blue). Scale bar equals 10 μm.

(I and J) Cell-cycle activation does not accompany direct induction of apoptosis by reaper overexpression. 24 hr after induction of reaper, many neurons are found to be TUNEL positive (I, arrows), but the brain is completely immunonegative for PCNA (J). Scale bar equals 10 μm.

also coexpressed a transgenic Tsc2 construct lacking inhibitory Akt phosphorylation sites ($Tsc2^{\Delta AKT}$; [30]) with tau and found a marked reduction in TUNEL staining (Figure 5D) and vacuolization (not shown). In addition, the tau-induced rough eye was dominantly suppressed by $Tsc2^{\Delta AKT}$ expression (Figure S3E) and by Rheb, TOR, S6k, and eIF4e loss-of-function alleles (Figures S3F and S3G; not shown), an effect that could be augmented by coexpressing Tsc transgenes or by removing a single copy of *Cdk4* (Figures S3H and S3I). We conclude that both genetic and pharmacologic inhibition of the TOR pathway rescues neurons from tau-mediated toxicity.

To establish a requirement for TOR signaling in tau-induced neurotoxicity, we overexpressed tau in a background null for specific TOR pathway components. A P element insertion (*fs(3)07084*) has been described in the 5′ noncoding region of the *S6k* gene, the excision of which results in a complete null allele with severe growth restriction of homozygotes ($S6k^{l-1}$; [31]). In flies transheterozygous for the insertion and excision, S6k activity (measured by P-S6k) was not detectable (not shown) and tau toxicity was substantially reduced (Figure 5F). While flies null for TOR do not survive to adulthood, certain transheterozygous allelic combinations are viable. Strikingly, tau toxicity was minimal in a genetic background in which TOR activity was reduced by approximately 75% (S.O., unpublished data; Figure 5G), and flies were obviously reduced in size (Figure S3B). These data indicate that TOR signaling is required for tau-induced neurotoxicity.

While overexpressing TOR itself paradoxically downregulates TOR signaling [32], overexpressing Rheb effectively activates the pathway [28, 29]. Coexpression of a Rheb transgene ($Rheb^{AV4}$; [29]) with tau strikingly enhanced the tau-induced rough eye (Figure 6B). Expression of $Rheb^{AV4}$ alone gave an overgrowth phenotype with mild roughness (Figure S3C). TOR modulation similarly modified the tau^{WT}-induced rough eye (Figure S5).

To determine the mechanism of TOR-dependent modification of tau toxicity, we first established that TOR modulation did not alter tau levels or tau phosphorylation (Figure 5H; data not shown). Notably, however, Rheb-dependent enhancement of tau toxicity was blocked by concomitant cell-cycle inhibition, either dominantly by removing one copy of *Cdk4* ($Cdk4^{3}$; Figure 6C) or by coexpressing the cell-cycle inhibitors Dap and Rbf1 (Figure 6D). Furthermore, when TOR was ectopically activated in the fly brain, we found numerous PCNA-, PH3-, and TUNEL-positive neurons (Figures 6E–6G). Thus, TOR activation can lead to cell-cycle activation and apoptosis of postmitotic neurons in vivo. Taken together, these results are consistent with TOR signaling mediating tau-induced neurodegeneration via cell-cycle activation.

Apoptosis in Several Tau-Independent Neurodegeneration Models Is Cell Cycle Independent

We investigated tau-independent models of apoptotic neurodegeneration in flies to determine whether TOR-dependent cell-cycle activation invariably drives neurodegeneration in vivo. Expression of a mutant polyglutamine protein MJD (Machado Joseph Disease) with an expanded polyglutamine tract in the mushroom body of the fly brain led to progressive loss of cortical neurons and rarefaction of the neuropil (Figures 7A and 7B; [33, 34]). While neurodegeneration was apoptotic (Figure 7C), there were no PCNA-positive (Figure 7D) or PH3-positive (not shown) neurons. Furthermore, TOR and cell-cycle modifiers did not alter MJD-induced retinal degeneration (Figures 7E–7I; [33]). Thus, TOR signaling and cell-cycle activation do not mediate MJD-78-dependent neurodegeneration in flies. Furthermore, we found no evidence of cell-cycle activation in a fly model

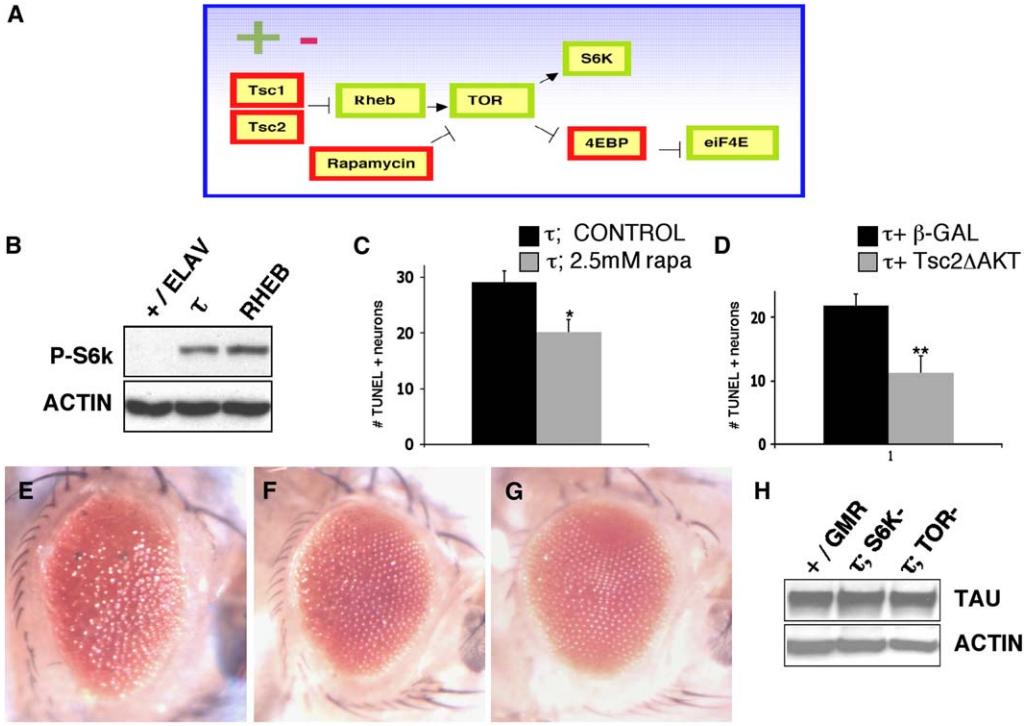

Figure 5. TOR Signaling Mediates Tau-Induced Neurodegeneration

(A) In *Drosophila*, as in mammalian cells, TOR is activated by Rheb, which is negatively regulated by Tsc proteins. The downstream effectors of TOR are eIF4e and S6k.

(B) An upregulation of S6k phosphorylation at T398 (P-S6k) is induced by panneural expression of tau^{R406W} at 10 days. Phospho-S6k^{T398} appears as a band at 70 kDa and is prominently upregulated in flies expressing *UAS-Rheb*. Driver: *ELAV-GAL4*.

(C and D) Neurodegeneration in the brain of 10-day-old flies, quantified by counting TUNEL-positive neurons, is suppressed by feeding flies the TOR-specific inhibitor rapamycin (C) or by coexpressing a constitutively active *Tsc2* transgene (D) (unpaired t test; *$p < 0.05$; **$p < 0.01$; error bars: ± SEM).

(E–G) TOR signaling is required for tau^{V337M}-induced neurotoxicity. The tau^{V337M}-induced rough eye (E) is prevented in backgrounds heteroallelic for S6k (F) or TOR (G). Eye size reduction (F and G) is attributable to the substantial inhibition of growth in these genetic backgrounds (see Figure S3B).

(H) Modulation of TOR signaling does not alter tau levels. Total tau levels shown for genotypes in (E)–(G).

of Parkinson's disease (PD; [35]), despite detecting TUNEL-positive cells (not shown). These data indicate that apoptotic neurodegeneration does not generally occur via cell-cycle activation.

Discussion

Cell-Cycle Activation Causes Tau-Induced Neurodegeneration

In this study we established a causal relationship between cell-cycle activation and tau-induced neurodegeneration in vivo. Expression of both mutant and wild-type tau induced cell-cycle activation in our model (Figures 1 and 3), and genetic inhibition of the cell cycle substantially reduced tau-induced neurodegeneration in both the fly brain and retina (Figures 2 and 3). The issue of causality has previously been unresolved, although important studies have documented aberrant neuronal cell-cycle markers in human tauopathies and, more recently, in a mouse tauopathy model [36]. Our data also provide in vivo support for key experiments in cell-culture systems that have demonstrated cell cycle-dependent apoptosis in a variety of neurotoxic paradigms [4, 8, 24, 37, 38]. Previous reports have implicated cell-cycle activation in rodent models of stroke and head trauma, although these studies have largely relied upon pharmacologic inhibition of the cell cycle by Cdk inhibitor drugs [39, 40]. Cdk inhibitors target several non-cell-cycle kinase targets, including GSK-3 and Cdk5 [23], that have also been implicated in cell survival and tau phosphorylation. Indeed, while Cdk inhibitors were recently shown to be neuroprotective in a toxic mouse model of PD, this effect was found to be more attributable to inhibition of Cdk5 than to inhibition of cell cycle-related kinases [41].

In this study, we demonstrated PCNA-, PH3-, and TUNEL-positive neurons in our tauopathy model (Figure 1) and concluded that cell-cycle activation and apoptosis accompanied neurodegeneration. While expression of PCNA and PH3 have been described in processes other than cell division (DNA repair and immediate early gene responses, respectively), our genetic data implicating multiple components of the cell cycle in tau-induced neurodegeneration strongly support a cell-cycle role in our model. While TUNEL-positive cell death may not always be apoptotic, previous reports showing that antiapoptotic genes, including IAP-1, block tau-induced neurodegeneration in flies [18, 25] support a role for apoptosis in this model. The role of apoptosis in tauopathies and animal tauopathy models remains

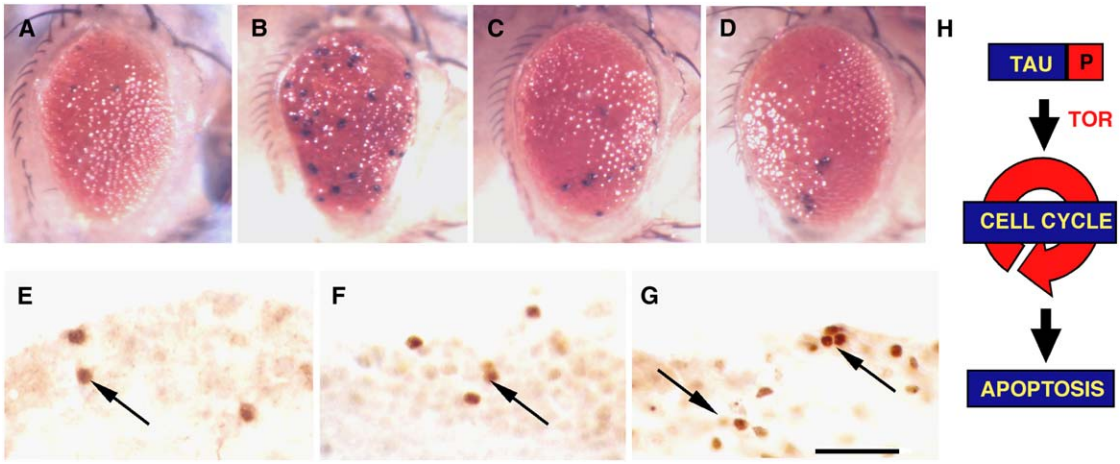

Figure 6. TOR Signaling Links Tau to Cell-Cycle Activation

(A–D) TOR activation enhances tau-induced neurotoxicity in a cell cycle-dependent manner. Rheb coexpression (B) enhances the tau-induced rough eye (A). This enhancement is blocked by concomitant cell-cycle inhibition, either by removing one copy of *Cdk4* (C) or by coexpressing Dap and Rbf1 (D).

(E–G) Expression of Rheb in the brain induces cell-cycle activation and neurodegeneration. Rheb induces PCNA expression (E, arrow), PH3 expression (F, arrow), and positive TUNEL staining (G, arrows) in neurons. Flies are aged to 30 days. Scale bar equals 10 μm.

(H) A model for cell-cycle activation in tau-mediated neurodegeneration suggested by our data. Cell-cycle activation occurs downstream of tau phosphorylation and upstream of apoptosis, and TOR links tau to the cell cycle.

controversial, however. Recently, both apoptotic and nonapoptotic neurodegeneration were described in a mouse tauopathy model [36], and we cannot rule out the possibility that nonapoptotic forms of cell death occur in the fly model also.

Cell-Cycle Activation Is Downstream of Tau Phosphorylation and Directly Leads to Apoptosis

The relationship between cell-cycle activation and tau phosphorylation has previously been unclear. Since Cdks are proline-directed kinases known to phosphorylate tau in vitro, cell-cycle activation could mediate neurodegeneration by directly phosphorylating tau. Indeed, several serine and threonine residues of tau are substrates for Cdks in vitro, and mitosis in cultured proliferating cells is associated with tau phosphorylation at these sites [26]. Second, cell-cycle activation could be downstream of phosphorylation and directly lead to apoptosis in two plausible ways. First, forcing differentiated cells to enter a cell cycle could directly lead to

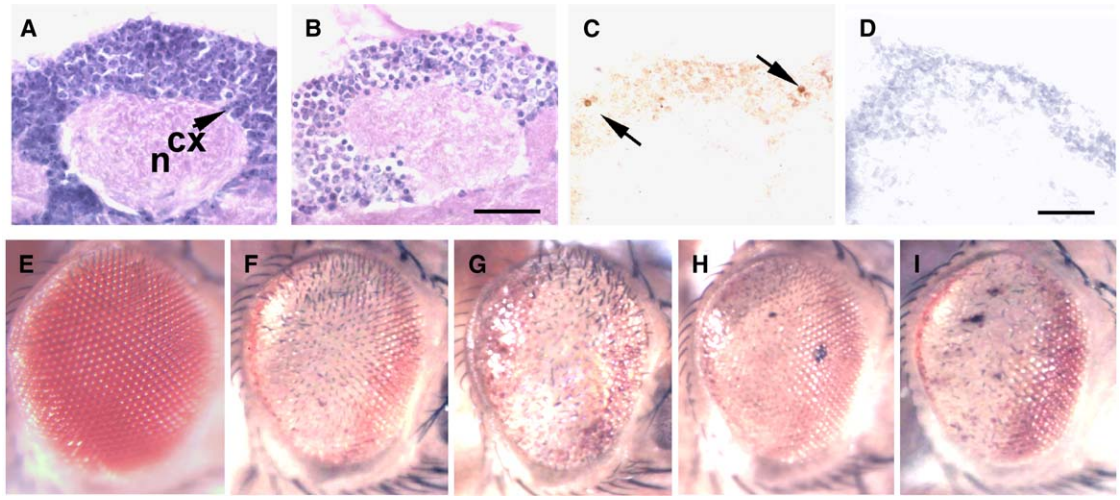

Figure 7. TOR-Mediated Cell-Cycle Activation Does Not Mediate MJD-Induced Neurodegeneration

(A–D) Expression of MJD-78Q in the brain induces apoptotic neurodegeneration in the absence of cell-cycle activation. The normal appearance of mushroom body cortex (cx) and neuropil (n) in control fly ([A]; genotype: *30Y-GAL4/+*) is altered by expressing MJD-78Q (B–D), which induces cortical neuron loss and neuropil rarefaction (B). Neurons are TUNEL positive (C, arrows) but are completely negative for PCNA (D). A blue (hematoxylin) nuclear counterstain is used in (D). Scale bars equal 10 μm.

(E–I) Cell-cycle or TOR pathway manipulation does not alter the MJD-78Q-dependent rough eye. Normal eye pigmentation and external morphology (E) is disrupted by expression of MJD-78Q (F). Coexpression of $Rheb^{AV4}$ (G), $Tsc2^{\Delta Akt}$ (H), or Dap and Rbf1 (I), modifiers that strongly modify the tau-induced rough eye, do not modify the MJD-78Q-induced rough eye.

apoptosis via an aborted attempt to replicate damaged DNA. Such a mechanism may be particularly relevant to postmitotic neurons that are known to have a limited capability for DNA repair [42]. Alternatively, it is possible that cell-cycle mediators, including E2F1 and Cdk1, may subserve dual functions as direct mediators of neuronal apoptosis [37, 43].

Our data support a role for cell-cycle activation downstream of tau phosphorylation and directly preceding apoptosis. First, cell-cycle markers often immunolocalized to areas characterized histologically by nuclear fragmentation and condensation (Figure 1F), suggesting a late role in neurodegeneration. Second, cell-cycle modulation dramatically modified tau-induced neurodegeneration without altering tau phosphorylation at disease-associated epitopes that can be generated by Cdks in vitro (Figure 3I). In contrast, pseudophosphorylation of tau (Figure 3J) or reducing tau phosphorylation in a *sgg* mutant background (Figure S2) directly increased and decreased cell-cycle activation, respectively. Third, cell-cycle modulation could still enhance toxicity of tau^{E14}, a pseudophosphorylated construct in which all Ser-Pro and Thr-Pro target sites are mutated to glutamate (Figures 3E–3H). Fourth, double labeling of brains of tau-expressing flies for PH3 and tau phosphoepitopes (Figure 3K) revealed that >90% PH3-positive neurons were phosphoepitope positive, even for relatively restricted epitopes such as AT-180 (20% of all neurons). Finally, we showed that cell-cycle activation, in the absence of transgenic tau, could directly lead to apoptosis of postmitotic neurons in vivo (Figures 4A–4H), supporting the possibility that cell-cycle activation could directly transduce tau-induced apoptosis.

TOR Signaling Mediates Tau-Induced Neurodegeneration via Cell-Cycle Activation

The mechanisms through which cell cycle becomes activated in tauopathies have not been defined. Markers that could represent aberrant mitogenic signaling are aberrantly expressed in these diseases, including markers of MAP kinase activation [44], classic oncogenic pathways such as Src [45] and c-Myc [46], and TOR activation [10–12]. However, the expression of isolated markers, while interesting, establishes neither the importance of a particular pathway as a whole nor whether any of these pathways are able to reactivate cell cycle in postmitotic neurons or lead to neurodegeneration in vivo. In this study, we showed that TOR activation occurred in our tauopathy model (Figure 5B), recapitulating a similar finding in AD [10], and was furthermore required for neurodegeneration (Figures 5C–5G). Since TOR-dependent enhancement of tau toxicity was blocked by concomitant cell-cycle inhibition, and ectopic TOR activation led to neuronal cell-cycle activation and apoptosis in the adult fly brain (Figure 6), our data indicate that TOR signaling mediates tau-induced neurodegeneration via cell-cycle activation. The relationship between tau, TOR, and cell-cycle activation, however, may be complex. In the retina, for example, TOR activation in the absence of transgenic tau results in an enlarged eye, whereas tau expression results in a small, rough eye. It is plausible that these phenotypic differences may be related to tau-induced pathogenic events that occur upstream of TOR activation in our model. Also, while our data strongly link TOR to cell-cycle activation in our model, other TOR-dependent mechanisms of neurotoxicity cannot be ruled out. For example, TOR activation could theoretically enhance neurodegeneration by inhibiting autophagy, although the rescue of tau toxicity by loss of S6k (Figure 5F) would argue against this possibility since S6k is an activator of autophagy in flies [47].

Aging is a significant risk factor for tauopathies. Interestingly, TOR inhibition is known to prolong lifespan in *Drosophila* [14], and our data thus directly link an aging-related signal transduction pathway to tau-induced neurodegeneration. Furthermore, withdrawal of amino acids in vitro or starvation in vivo results in inhibition of TOR signaling [13], potentially offering a molecular mechanism for the neuroprotection reported in human studies by caloric restriction [48].

Cell-Cycle Activation May Be Specific to Tau-Induced Neurodegeneration

In this study, we investigated the role of cell-cycle activation in tau-mediated neurodegeneration because aberrant expression of cell-cycle markers is best described for tauopathies and AD. For example, one comprehensive study found upregulation of cell-cycle markers in AD and in a cohort of sporadic and inherited tauopathies but not in other diseases including PD, Huntington's disease, amyotrophic lateral sclerosis (ALS), or multiinfarct dementia [3]. However, others have reported cell-cycle marker upregulation in the context of spinal cord injury, ALS, and PD [8]. In addition, neuronal expression of cell-cycle markers has been described in several cell-culture paradigms of neurotoxicity (see above) and in mouse models of ataxia [49, 50], leading to the speculation that cell-cycle activation might be a universal mechanism for neurodegeneration, perhaps related to oxidative stress [8, 50]. In our study, however, we did not find evidence of cell-cycle activation in fly models of either MJD (Figure 7) or PD (not shown), despite the presence of apoptotic neurodegeneration, and cell-cycle modulation did not modify MJD-induced neurodegeneration (Figure 7). These data implicate distinct mechanisms for neurodegeneration in different neurodegenerative diseases, consistent with recent findings that forward genetic screen modifiers differ between polyglutamine-, synuclein-, and tau-induced neurodegeneration [51, 52].

In summary, our results indicate a common effector pathway and potentially common therapeutic strategies for cancer and tauopathy, two major causes of age-related morbidity and mortality. We delineate the TOR signaling pathway, a known regulator of lifespan, as a required mediator of tau-dependent neurodegeneration in vivo. Our results provide a rationale for the assessment of TOR and cell-cycle inhibitors as potential therapeutic strategies in tauopathies and AD.

Experimental Procedures

Fly Stocks, Genetics

All fly crosses and experiments were performed at 25°C. Flies were age and sex matched in assessing modification of brain and eye toxicity. The human wild-type and mutant tau transgenic flies were previously described [17]. TOR^{2L7} and TOR^{2L15} will be described in detail elsewhere (hypomorphic missense alleles; S.O., submitted).

Vector pBS-htau24 [17] served as a template for the generation of tau^{E14}. The codons for T111, T153, S175, T181, S199, S202, T205, T212, T217, T231, S235, S396, S404, and S422 were mutated to glutamate (GAA or GAG) with the QuikChange site-directed mutagenesis kit (Stratagene), and the resulting pBS-htau24^{E14} was subcloned into the pUAST vector to generate *UAS-tau^{E14}*. Lines with expression level equivalent to tau^{WT} were determined by quantitative Western blot.

Drivers used were *ELAV-GAL4* (panneural), *GMR-GAL4* (retinal). *30Y-GAL4* (mushroom body) and *ELAV-GeneSwitch* (panneural, inducible [27]). Flies were obtained from the Bloomington *Drosophila* Stock Center (*ELAV-GAL4, GMR-GAL4, UAS-rpr*, *CycB3^{L6540}*, *CycA^{C8LR1}*, *Cdk4^{3}*, *eIF4e^{07238}*, *S6k^{07084}*, *S6k^{l-1}*, *TOR^{k17004}*, *Df(E2F1)$^{(3R)e-BS2}$*, *Df(E2F1)$^{(3R)e-N19/TM2}$*, *E2F1^{07172}*, *sgg^{1}*) or were generous gifts from N. Bonini (*UAS-MJD-78Q*), W. Cha (*UAS-Cdk1^{E51Q}* [22]), W. Du (*UAS-Rbf^{280}*), N. Dyson (*UAS-E2F1, UAS-DP, UAS-Rbf1*), P. Gallant (*UAS-Rheb$^{1.1}$*), E. Hafen (*Rheb^{2D1}*), I.K. Hariharan (*UAS-dap* [21], *UAS-p21, UAS-Tsc1*, *UAS-Tsc2*), K. Kaiser (*30Y-GAL4*), H. Keshishian (*ELAV-GeneSwitch*), C. Lehner (*UAS-Cdk1, UAS-Cdk2, UAS-Cdk4, UAS-CycA, UAS-CycB, UAS-CycD*), T. Orr-Weaver (*E2F^{91}*), F. Tamanoi (*Rheb^{AV4}* [29]), and T. Xu (*UAS-Tsc1, UAS-Tsc2$^{\Delta Akt}$*). Note that *Cdk1* is identical to *cdc2*.

Complete genotypes can be found in the Supplemental Data.

Sectioning, Immunostaining, and Histology

Adult flies were fixed in formalin at 1 or 10 days and embedded in paraffin. Serial frontal 4 μm sections including the entire brain were prepared. Antigen retrieval was performed by microwaving in sodium citrate buffer. Immunostaining was performed with an avidin-biotin-peroxidase complex (ABC) method (Vector Laboratories) or with secondary antibodies coupled to Alexa Fluor 488 or Alexa Fluor 555. For quantification of PH3/tau phosphoepitope double labeling, alkaline phosphatase-conjugated secondary antibodies were used and histochemical detection performed with Vector Red (Vector Laboratories) and DAB. Primary antibodies included anti-PCNA (1:500; Biomeda), -PH3 (1:500; Upstate), -AT8 (1:1000; Innogenetics), -PHF-1 (1:200; a generous gift from P. Davies), and -AT180 (1:500; Innogenetics). Secondary antibodies were used at 1:200 dilution. Where noted in the figures, nuclei were counterstained with hematoxylin. To assess morphology, sections were stained with hematoxylin and eosin under a standard protocol. For PCNA quantification (Figure 3; Figure S2), at least four brains were used per genotype and the number of PCNA-positive neurons/neuronal clusters were summed across the whole brain. Statistical significance was established as for TUNEL staining (see below). We assessed PH3/tau phosphoepitope colocalization (p10) in 10 frontal sections through the central body complex from at least 6 hemibrains. The overall prevalence of tau phosphoepitopes was quantified in the calyx of the mushroom body in 8 hemisections (4 separate brains; average of 135 cells/hemisection).

TUNEL Staining

Neuronal apoptosis was detected with the TUNEL assay with a commercially available kit (TdT FragEl, Oncogene). Where noted, nuclei were counterstained with methyl green. Neurodegeneration was quantified in 10-day-old (Figures 2 and 5) or 30-day-old (Figure 3) flies by counting the number of TUNEL-positive cells per hemibrain in consecutive frontal sections between the ellipsoid body anteriorly and the mushroom body posteriorly. At least eight hemibrains were examined per time point for each genotype. Statistical significance was established by one-way ANOVA with post hoc test for multiple comparisons (Student-Neuman-Keuls; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). An unpaired t test was used for the single comparison in the $tau^{E14,}$ rapamycin, $Tsc2^{\Delta AKT}$, and Sgg experiments (Figures 3K, 5C, and 5D and Figure S2, respectively; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

Western Blot

Heads from adult flies at 1 day posteclosion were homogenized, separated by SDS-PAGE, and immunoblotted with one of the following antibodies: anti-tau (Dako; 1:1 million); monoclonal antibodies to AT8, AT180, PHF-1 as described above at 1:100,000, 1:1,000, and 1:100,000, respectively; anti-AT100 (Innogenetics; 1:1000); anti-phosphoS6k^{T398} (*Drosophila*-specific; Cell Signaling; 1:1000); anti-total S6K (C-terminal monoclonal; 1:500; a generous gift from G. Thomas); or anti-actin (20-66; Sigma; 1:50,000). The appropriate secondary antibody was applied at 1:50,000 diultion and signals were detected by chemiluminescence (Pierce). Boiling (5 min, PBS) was used for antigen retrieval prior to blocking in the case of AT180 and AT100. At least three separate experiments were used for quantitation by ImageJ (NIH). For Figure 3I, one-way ANOVA with Student-Neuman-Keuls post hoc test for multiple comparisons was used to test for statistically significant differences.

Drug Feeding

Olomoucine

1, 5, or 10 mM olomoucine (Sigma) in 10% DMSO was mixed in a yeast paste and fed to flies for 10 days after eclosion. Food was changed every second day. Control flies were fed 10% DMSO alone. See Figure 2.

RU486

For induction of E2F1-DP expression with the RU486-inducible *ELAV-GeneSwitch* driver (Figure 4; [27]), we fed flies RU486 (10 mg/ml; Sigma) for 10 days after eclosion in instant *Drosophila* medium (Carolina). Controls were fed the same concentration of drug for 10 days. For reaper induction, we fed 1-day-old flies RU486 for 24 hr. Control flies were drug-free 1-day-old flies of the same genotype.

Rapamycin

2.5 mM rapamycin (Sigma) in 2.5% DMSO was mixed in a yeast paste and fed to flies for 10 days after eclosion. Control flies were fed 2.5% DMSO alone. Food was changed every second day. See Figure 5.

Supplemental Data

Supplemental Data include five figures and Supplemental Experimental Procedures and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/3/230/DC1/.

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