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Pathogenic Forms of Tau Inhibit Kinesin-Dependent Axonal Transport Through a Tau-Dependent Mechanism Involving Activation of Axonal Phosphotransferases

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Abstract

Aggregated filamentous forms of hyperphosphorylated tau (a microtubule-associated protein) represent pathological hallmarks of Alzheimer's disease (AD) and other tauopathies. While axonal transport dysfunction is thought to represent a primary pathogenic factor in AD and other neurodegenerative diseases, the direct molecular link between pathogenic forms of tau and deficits in axonal transport remain unclear. Recently, we demonstrated that filamentous, but not soluble, forms of wild-type tau inhibit anterograde, kinesin-based fast axonal transport (FAT) by activating axonal protein phosphatase 1 (PP1) and glycogen synthase kinase 3 (GSK3), independent of microtubule binding. Here, we demonstrate that amino acids 2-18 of tau, comprising a phosphatase-activating domain (PAD), are necessary and sufficient for activation of this pathway in axoplasms isolated from squid giant axons. Various pathogenic forms of tau displaying increased exposure of PAD inhibited anterograde FAT in squid axoplasm. Importantly, immunohistochemical studies using a novel PAD-specific monoclonal antibody in human postmortem tissue indicated that increased PAD exposure represents an early pathogenic event in AD that closely associates in time with AT8 immunoreactivity, an early marker of pathological tau. We propose a model of pathogenesis in which disease-associated changes in tau conformation lead to increased exposure of PAD, activation of PP1-GSK3 and inhibition of FAT. Results from these studies reveal a novel role for tau in modulating axonal phosphotransferases and provide a molecular basis for a toxic gain-of-function associated with pathogenic forms of tau.

Introduction

Filamentous aggregates of the microtubule-associated protein, tau, represent major pathological components in Alzheimer's disease (AD) and other tauopathies (Grundke-Iqbal et al., 1986; Wood et al., 1986), and correlate with progressive cognitive decline in AD (Arriagada et al., 1992; Giannakopoulos et al., 2003). The identification of tau mutations in hereditary tauopathies directly link tau to neurodegeneration (Goedert and Jakes, 2005). However, the molecular mechanisms behind tau-mediated toxicity remain unclear.

Axonal transport dysfunction is implicated as a critical pathogenic factor in AD and other neurodegenerative diseases (Roy et al., 2005; Morfini et al., 2009). Experiments in isolated squid axoplasm revealed that filamentous WT tau selectively inhibited kinesin-based anterograde fast axonal transport (FAT) at physiological levels, while soluble WT tau monomers did not, even at concentrations >10-fold higher than physiological levels (LaPointe et al., 2009; Morfini et al., 2009). The toxic effect of filamentous tau on anterograde FAT involved activation of protein phosphatase 1 (PP1) and glycogen synthase kinase 3 (GSK3) (LaPointe et al., 2009). Deletion experiments suggested that this effect required amino acids 2-18 of tau (LaPointe et al., 2009). However, these studies did not explain why monomeric, soluble tau did not inhibit FAT.

Findings on the conformation of soluble tau provided a potential explanation for this apparent paradox. Originally, tau was considered a "natively unfolded" protein with little secondary structure in solution (Schweers et al., 1994). However, recent fluorescence resonance energy transfer studies suggest soluble WT tau monomers adopt a "paperclip" conformation involving close interactions of the C-terminus with the microtubule binding repeats (MTBRs) and the N-terminus with the C-terminus (Jeganathan et al., 2006). Pathological forms of tau appear to alter the paperclip

conformation. For example, the AT8 phosphoepitope (Ser199/Ser202/Thr205) found in AD and other tauopathies reduced folding of the N-terminus into the paperclip conformation (Jeganathan et al., 2008). Similarly, a tau mutation in a patient with inherited frontotemporal dementia (FTD) results in deletion of the “hinge” region that is crucial for N-terminal folding (Rovelet-Lecrux et al., 2009), rendering this mutant tau protein incapable of assuming the paperclip conformation. These observations suggest that disease-associated tau modifications disrupt the paperclip conformation, but molecular mechanisms linking disruptions in tau conformation to inhibition of FAT remained unknown.

We present evidence indicating that disease-associated modifications in tau that impair the paperclip conformation inhibit anterograde FAT. Results from our experiments suggest that amino acids 2-18 of tau, a domain we refer to as the phosphatase-activating domain (PAD), represent a biologically active protein motif capable of activating the PP1-GSK3 pathway. The relevance of these findings to AD was validated using a novel PAD-specific antibody to document increased PAD exposure early in the course of disease. Based on these results, we propose a model of pathogenesis where modifications in tau, including aggregation, hyperphosphorylation and FTD-associated mutation all result in increased PAD exposure, activation of the PP1-GSK3 pathway and inhibition of anterograde FAT. Collectively, our findings provide a common molecular basis for the toxic gain-of-function mechanism associated with biochemically distinct disease-related modifications of tau.

Materials and Methods

Reagents. The following reagents were purchased from commercial vendors: okadaic acid (EMD, 495604) and inhibitor 2 (I-2; EMD, 539638); PAD peptide (amino acid 2-18) and scrambled PAD peptide (both >98% purity) (GenScript, Piscataway, NJ); Complete, EDTA-free Protease Inhibitor tablets (Roche, 11873580001). ING-135 (Kozikowski et al., 2007) was a generous gift from Dr. A. Kozikowski (UIC).

Recombinant Tau Proteins. Tau proteins are numbered according to the largest adult human isoform (ht40; 441 amino acids) in the CNS. Full-length ht40 (WT tau) was generated from previously described pT7c plasmids (Figure 1A) (LaPointe et al., 2009). Site-directed mutagenesis (Stratagene, QuickChange II Kit, 200524) was used to create point mutations in full-length tau constructs. Serine 199, Ser202, and Thr205 were mutated to glutamic acid (E) to create the AT8 pseudophosphorylated mutant protein (AT8 tau; Figure 1B). Amino acids 144-273 (Δ 144-273 tau; Figure 1C) were deleted by inserting EcoRV restriction sites flanking the appropriate region of the cDNA. Following EcoRV digestion and T4 ligation (New England Biolabs; according to manufacturer's instructions), the remaining EcoRV site was removed via deletion using the site-directed mutagenesis kit described above. The 6D tau and 6P tau constructs were generated from previously described pT7c plasmid cDNAs (Figure 1D and 1E) (LaPointe et al., 2009; Luo et al., 2004). A 6D protein with amino acids 2-18 deleted (Δ 2-18 6D tau; Figure 1F) was created by restriction digestion and ligation of 6D and Δ 2-18 tau cDNAs, both of which have been described previously (LaPointe et al., 2009). The tau cDNA plasmid constructs were amplified in XL-1 blue supercompetent *E. coli*, purified with the Qiagen Miniprep kit, and cDNA sequences were verified by DNA sequencing. T7 express supercompetent *E. coli* cells were used to express tau constructs and the proteins were

purified through His-tag high affinity resin and size exclusion chromatography as previously described (Carmel et al., 1996).

Squid Axoplasm Motility Assay. Freshly extruded axoplasms from squid giant axons (*Loligo pealii*; Marine Biological Laboratory, Woods Hole, MA) were used to measure axonal transport as described previously (LaPointe et al., 2009; Morfini et al., 2007). Tau proteins were diluted in X/2 buffer (175mM potassium aspartate, 65mM taurine, 35mM betaine, 25mM glycine, 10mM HEPES, 6.5mM MgCl₂, 5mM EGTA, 1.5mM CaCl₂, 0.5mM glucose, 10mM ATP, pH 7.4) and perfused into isolated axoplasms at a final concentration of 2μM. Use of tau proteins at 2μM was specifically chosen since this is within the physiological levels of tau in brains (Alonso, et al., 1996; King et al., 2006) and is within the physiological range of tau monomer to tubulin dimer ratios (Drubin et al., 1985; LaPointe et al., 2009; Morfini et al., 2007). A Zeiss Axiomat microscope equipped with a 100X objective (1.3 N.A.) and DIC optics, equipped for digital video processing and analysis was used to visualize vesicle motility (Brady et al., 1993). Anterograde and retrograde transport were measured by matching vesicle speed to calibrated cursors over 50 minutes as described previously (Brady et al., 1993; Morfini et al., 2002b).

Preparation of ³²P-radiolabelled Phosphatase Substrate and Phosphatase Assay in Squid Axoplasm. Recombinant GST-c-Jun (75μg) was incubated with 10μg recombinant p38α (a generous gift from Dr. D. Bosco, Univ. of Mass.) in the presence of 100μM ³²P-radiolabeled ATP in a 20μl volume. After 1 hour at 30° C, the resulting ³²P-c-Jun samples were stored at -20° C until used in phosphatase assays.

First, a control experiment was run to confirm that endogenous axoplasmic phosphatases could dephosphorylate ^{32}P -c-Jun. Squid axoplasm was incubated with ^{32}P -c-Jun (1 μg) for 0 minutes (Input) or 30 minutes (control), and with okadaic acid (1 μM) or I-2 (400nM) for 30 minutes. To determine whether PAD affects phosphatase activity, sister axoplasms were extruded on cover glasses and perfused with 35 μl of either PAD or scrambled PAD peptides (10 μM each) in buffer X/2 supplemented with 1 mM ATP plus 1 μg of ^{32}P -cJun. A 4 μl aliquot of perfusate was removed at 10, 20 and 30 minutes, and transferred to tubes containing 9 μl of sample buffer. Samples were run on SDS-PAGE, the gels were dried and ^{32}P incorporation in GST-cJun was analyzed with the Typhoon phosphorimager.

Kinase Assay in Isolated Squid Axoplasm. GSK3 assays were modified from Morfini et al. (2007). Sister axoplasms were extruded on cover glasses and perfused with 30 μl of either PAD or scrambled PAD peptides (10 μM) in buffer X/2 supplemented with 1 mM ATP. After 45 minute incubation, 12 μl aliquots of perfusate containing solubilized axoplasmic proteins was removed from each axon, diluted to 80 μl with 20 mM HEPES, pH 7.4, 3 mM EDTA, 2 μM okadaic acid, and 1/80 protease inhibitor cocktail (Sigma, P8340), and kept on ice. Aliquots of diluted perfusates (20 μl) were supplemented with either 200 μM GSM (Upstate, 12-533) or with 50 μM MARCKS (Calbiochem, 454880) and the final volume adjusted to 32 μl with 20 mM HEPES, pH 7.4. Kinase reactions were started by adding 8 μL of 5X kinase buffer (100 mM HEPES, pH 7.4, 250 mM K aspartate, 50 mM MgCl_2 , and 500 μM of ^{32}P -radiolabeled ATP). After 30 min incubation at room temperature, aliquots were spotted on P81 cellulose paper in duplicate. Papers were washed three times in 100mM phosphoric acid and ^{32}P incorporation into peptides was measured by scintillation counting. GSK3 activity was

expressed as the ratio of incorporation between GSM and MARCKS peptide for each condition.

Monoclonal Antibody Production. Mouse monoclonal antibodies were raised against a synthetic PAD peptide (C²AEPRQEFVMEHDHAGTY¹⁸) conjugated to KLH (GenScript, Piscataway, NJ) as previously described (Reyes et al., 2008). Briefly, BALB/c female mice (Jackson laboratories, 001026) were immunized subcutaneously with peptides every 21 days over a five-month period; splenocytes were then removed and fused to SP2/o myeloma cells. Positive clones that reacted specifically with PAD by ELISA analysis (Reyes et al., 2008) were selected and then subcloned four times at limiting dilutions and isotyped (TNT1 is an IgG1). Antibody production was accomplished in a CELLline Classic 1000 bioreactor (Sartorius AG, DC-90005) prior to protein A purification at high pH. The sensitivity and specificity of the TNT1 antibody for PAD in tau was assessed using ELISAs and Western blotting (Figure 7A).

Tau Isolation from Human Brain Tissue. Fresh frozen frontal cortex from control (n=4) and severe AD (n=5) brains was obtained from the Brain Bank of the Cognitive Neurology and Alzheimer's Disease Center at Northwestern University. Heat stable/acid soluble tau was extracted based on protocols described before (Reyes et al., 2008). Sarkosyl insoluble tau (PHF) and sarkosyl soluble tau were obtained using methods similar to those in previous reports (Hasegawa et al., 1992).

Western and Dot Blots. For Western blot analysis, soluble and insoluble tau samples were separated on 10% SDS PAGE and transferred to nitrocellulose membranes as described previously (Reyes et al., 2008). For dot blots, soluble and insoluble tau samples were

spotted directly onto nitrocellulose membranes (LaPointe et al., 2009). Both Western and dot blot membranes were blocked with 2% non-fat dry milk in tris buffered saline (TBS, pH 7.4) and incubated in primary antibodies overnight at 4°C. The Tau12 (Horowitz et al., 2004) and TNT1 mouse monoclonal antibodies were diluted from 1mg/ml purified stock solutions to 1:500,000 and 1:300,000, respectively. The rabbit polyclonal antibody, R1 tau (Berry et al., 2004), was diluted 1:300,000 from a 1mg/ml stock. After rinsing, the membranes were incubated in peroxidase-conjugated horse anti-mouse secondary antibody (Vector Labs, PI-2000) for 1 hour at room temperature. Reactivity was visualized using ECL substrate (Pierce, PI32106) and Western blots were visualized on Kodak Bio-max XAR film, while dot blots were imaged on the Kodak imaging station 4000R for quantification of signal intensity. Data are expressed as the ratio of TNT1:Tau12 intensity.

TNT1 Immunohistochemistry. Tissue sections (40µm thick) from the entorhinal cortex, hippocampus, inferior temporal gyrus and superior temporal gyrus of control (Braak stages I-II; n=4) and severe AD (Braak stages V-VI; n=4) cases were obtained through the Cognitive Neurology and Alzheimer's Disease Center at Northwestern University. All sections were stained simultaneously to reduce variability in tissue processing. Sections were processed as previously described (Kanaan et al., 2007). The TNT1 primary antibody was diluted 1:400,000 (from 1mg/ml stock) and incubated with the tissue sections overnight at 4° C. The tissue was incubated in biotinylated goat anti-mouse secondary antibody (Vector, BA-9200, diluted 1:500) for 2 hours, followed by incubation in ABC solution (Vector, PK-6100, according to manufacturer's instructions) for 1 hour. The staining was developed with 3,3'-diaminobenzidine (Sigma, D5637). All steps were done at room temperature unless otherwise noted and each step was followed

by 6 x 10 minute rinses in TBS-0.5% Triton X-100. After developing the stain, the tissue sections were mounted and coverslipped. Images were taken using a Nikon Eclipse 90i microscope equipped with a DS-Ri1 camera and Nikon Elements AR 3.10 software. Figures were generated using Adobe Photoshop.

TNT1 and AT8 Double-label Immunofluorescence. Tissue sections (as described above) from the hippocampus of control (Braak stages I-II; n=4), mild AD (Braak stages III-IV; n=5) and severe AD (Braak stages V-VI; n=4) cases were processed for double-label immunofluorescence using TNT1 and the AT8 antibody (Biernat et al., 1992; Goedert et al., 1995). All sections were stained simultaneously using similar methods as previously described (Kanaan et al., 2007) to reduce variability in tissue processing. The TNT1 primary antibody was diluted 1:40,000 (from 1mg/ml stock) and incubated with the tissue sections overnight at 4° C. The tissue was incubated in Alexa Fluor 594 goat anti-mouse IgG1 γ chain-specific secondary antibody (Invitrogen, A21125, diluted 1:500) for 2 hours, followed by blocking uncomplexed secondary antibody sites with 10% mouse serum for 1 hour. Then the sections were incubated overnight at 4° C with biotinylated AT8 antibody (Thermo Scientific, MN1020B) diluted at 1:1000. The AT8 antibody was labeled with Alexa Fluor 488 conjugated streptavidin (Invitrogen, S11223, diluted 1:500) for 2 hours. Sections were mounted and coverslipped using Vectashield Hardest (Vector, H-1400). All steps were performed at room temperature unless otherwise noted and each step was followed by 6 x 10 minute rinses in TBS-0.5% Triton X-100. Images were taken using a fluorescence equipped Nikon Eclipse 90i microscope with a Q-imaging Retiga-SRV Fast1394 camera and Nikon Elements AR 3.10 software (with live de-blur function). Figures were generated using Adobe Photoshop.

Statistics. Each experiment was repeated at least three times (see figures for n values). Comparisons of vesicle motility rates were made using an unpaired t-test or a one-way ANOVA as indicated. The WT tau monomer group was used as the control group since no changes in motility rates are observed in this group compared to unperfused axoplasms (LaPointe et al., 2009; Morfini et al., 2007). When overall significance was reached, the Student-Newman-Keuls *post-hoc* analysis was used to make all possible comparisons. Kinase assay with results were compared using a paired t-test (paired by sister axoplasms). Significance was set at $p < 0.01$ or $p < 0.05$ as indicated. Sigmastat software (Systat Software, Inc., San Jose, CA) was used for all statistical tests.

Results

6D and 6P Tau Isoforms Inhibit Anterograde FAT

The isolated squid axoplasm is an *ex vivo* model system that allows for direct, quantitative evaluation of different effectors on anterograde (conventional kinesin-dependent) and retrograde (cytoplasmic dynein-dependent) FAT rates by video microscopy. When isolated axoplasm is perfused with tau filaments, but not soluble tau monomers, there is a selective inhibition of anterograde FAT through an N-terminal mediated mechanism involving activation of a PP1-GSK3 pathway, phosphorylation of kinesin light chains and dissociation of kinesin-1 from its transported cargoes (LaPointe et al., 2009; Morfini et al., 2004).

To explore the N-terminal dependence of tau-mediated FAT inhibition we utilized the non-canonical N-terminal 6D and 6P tau isoforms (Figure 1D and 1E). Both 6D and 6P are identical to WT-tau from amino acid 1-143, but lack the proline-rich region, the MTBRs and C-terminus of full-length tau and each protein contains a unique C-terminal 11 amino acid sequence (Luo et al., 2004; Andreadis, 2005). Thus, the 6D/6P isoforms can be used as physiological tools for the evaluation of the N-terminus of tau in isolation from other functional domains and independent of microtubule binding or self-aggregation into filaments because both of these processes require the MTBRs.

Vesicle motility assays in isolated axoplasm confirmed and expanded our initial results with the N-terminal isoforms of tau. As previously reported (LaPointe et al., 2009; Morfini et al., 2007), perfusion of full-length WT tau monomers (2 μ M; Figure 1A) had no effect on FAT in squid axoplasm (Figure 2A), while 6D and 6P tau monomers (2 μ M) significantly inhibited anterograde FAT when compared to WT tau monomer (Figure 2B and 2C) or buffer controls (data not shown). Neither 6D nor 6P tau (Figure 2D) had an effect on retrograde FAT. Together, these data indicate that the proline rich

region, MTBRs and the C-terminus of full-length tau as well as the unique 11 amino acid C-termini of 6D and 6P do not play a role in inhibiting anterograde FAT. Since similar levels of FAT inhibition were obtained with 6D and 6P constructs (Figure 2C and 2D), which are identical in the first 143 amino acids, we chose to focus on the 6D tau for further experimentation.

6D Tau Inhibits FAT by Activating a PP1-GSK3 Signaling Cascade

Results from our previous work (LaPointe et al., 2009) prompted us to evaluate whether filamentous tau and 6D tau inhibited anterograde FAT through a common mechanism. To this end, we co-perfused axoplasms with 6D tau and pharmacological inhibitors of either PP1 or GSK3. Co-perfusion of 6D tau with Inhibitor-2 (I-2; 50nM), a specific PP1 inhibitor, completely prevented the effects of 6D tau on anterograde FAT (Figure 3A and 3C). Similarly, co-perfusion of 6D tau with okadaic acid (200nM), a less specific inhibitor of the major serine/threonine phosphatases (including both PP1 and PP2), prevented the inhibition of anterograde FAT elicited by 6D and 6P tau (data not shown). Furthermore, co-perfusion of 6D tau and the GSK3-specific pharmacological inhibitor, ING-135 (100nM) (Kozikowski et al., 2007; LaPointe et al., 2009), completely blocked the inhibitory effects of 6D tau on anterograde FAT (Figure 3B and 3C). Retrograde transport was not significantly affected in any experiment (Figure 3D). Together, these data demonstrate that, as posited for tau filaments (LaPointe et al., 2009), short N-terminal isoforms of tau inhibit anterograde FAT by a mechanism involving activation of PP1 and GSK3 that is independent of microtubule binding.

PAD is Necessary and Sufficient for Anterograde FAT Inhibition

Filaments composed of tau lacking amino acids 2-18 or PAD (Δ 2-18) did not inhibit anterograde FAT (LaPointe et al., 2009), suggesting that this domain plays a critical role in the activation of the PP1-GSK3 pathway. To determine whether this domain mediates the inhibitory effects of 6D tau monomer on FAT, axoplasms were perfused with a recombinant 6D tau protein lacking PAD (Δ 2-18 6D tau; 2 μ M; Figure 1F). As observed with Δ 2-18 tau filaments (LaPointe et al., 2009), monomeric Δ 2-18 6D tau showed no effect on FAT (Figure 4A and 4D), demonstrating that PAD is necessary for 6D tau-mediated inhibition of anterograde FAT.

We next sought to determine whether PAD is sufficient to induce anterograde FAT inhibition or whether other tau domains are also required. Isolated axoplasms were perfused with a synthetic PAD peptide (AEPRQEFVMEHDAGTY; Figure 1G) or with a scrambled control PAD peptide (AQEMDRVYEGEAPHTFE; Figure 1H) at the same concentration (2 μ M). The PAD peptide selectively inhibited anterograde FAT (Figure 4B and 4D), but not retrograde FAT (Figure 4B and 4E). In contrast, the scrambled PAD peptide had no effect on FAT in either direction (Figure 4C-E). Together, these data demonstrate that the PAD in the N-terminal region of tau is both necessary and sufficient to induce inhibition of anterograde FAT.

PAD Inhibits FAT through a PP1-GSK3 Signaling Cascade

We next evaluated whether FAT inhibition induced by the PAD peptide involved activation of PP1 and GSK3 as observed with 6D tau (above) and filamentous tau (LaPointe et al., 2009). Again, co-perfusion of the PAD peptide with either I-2 (50nM; Figure 5A) or ING-135 (100nM; Figure 5B) completely prevented anterograde FAT inhibition. Anterograde (Figure 5C) and retrograde (Figure 5D) FAT rates observed after

co-perfusion of the tau PAD peptide with either I-2 or ING-135 were indistinguishable from those observed after perfusion of soluble WT tau.

The effect of the PAD peptide on the activity of endogenous axoplasmic phosphatases was evaluated using a phosphatase activity assay (Figure 5E and 5F). In this assay, a recombinant GST-tagged c-Jun construct was phosphorylated with p38 kinase in the presence of $\gamma^{32}\text{P}$ -ATP for use as a phosphatase substrate. Isolated axoplasms were perfused with radiolabeled c-Jun (^{32}P -c-Jun), and aliquots of the perfusates were collected at various time points and separated by SDS-PAGE. Autoradiograph analysis showed a marked reduction in ^{32}P -c-Jun signal after 30 minutes incubation, indicating the action of axonal phosphatases. The reduction was completely abolished by okadaic acid and, to a lesser extent, by I-2, suggesting dephosphorylation of ^{32}P -c-Jun by axoplasmic serine-threonine phosphatases (Figure 5E). Next, two “sister” axoplasms were prepared from the same squid and each was perfused with ^{32}P -c-Jun and either the 2-18 or scrambled 2-18 peptides. Consistent with results from vesicle motility assays, ^{32}P -c-Jun was dephosphorylated to a greater extent in PAD peptide-perfused axoplasms than their scrambled peptide perfused counterpart (Figure 5F), suggesting that the PAD peptide induced activation of endogenous axoplasmic phosphatases.

Kinase activity assays were used to determine the effect of the PAD peptide on axoplasmic GSK3. Sister axoplasms were treated with PAD peptide or scrambled peptide, and then aliquots of perfusate containing axoplasmic proteins were incubated with ^{32}P -ATP and either a GSK3 (GSM peptide) or protein kinase C (MARCKS peptide) substrate. A trend of elevated GSK3 activity (~32%) relative to PKC activity ($p=0.28$, paired t-test, $n=3$) was observed for axoplasms incubated with the PAD peptide (mean= 4.9 ± 2.5), compared to those incubated with scrambled peptide (mean= 53.7 ± 1.7), which supports the results from vesicle motility assays. Reduced sensitivity to changes in

GSK3 activity may likely be a result of PAD affecting only a fraction of all available axoplasmic GSK3 enzymes. Collectively, these data indicate that PAD is both necessary and sufficient to inhibit anterograde FAT by activating the PP1-GSK3 cascade.

Disease-Associated Changes in Tau Cause Inhibition of FAT

Taken together, the above data suggest that increased exposure of PAD in the context of WT tau filaments and 6D/6P tau isoforms, results in PP1-GSK3 activation and anterograde FAT inhibition, whereas PAD exposure is limited in soluble WT tau. Consistent with this notion, a “paperclip” conformation has been shown for soluble, monomeric WT tau (Jeganathan et al., 2006). This conformation involves intramolecular interactions between the C-terminus and the MTBR, and between the extreme N-terminus (containing PAD) and the C-terminus. Folding of tau into the paperclip conformation may therefore limit exposure of PAD preventing it from activating the PP1-GSK3 cascade. This model predicts that disease-related tau modifications in monomeric tau that alter the paperclip conformation may result in abnormal exposure of PAD and should be toxic to FAT. To test this possibility, we evaluated the effects of two disease-associated forms of tau, termed “AT8 tau” and “ Δ 144-273 tau” (Figure 1B and 1C), on FAT.

Pseudophosphorylation of tau at the AT8 epitope (comprised of Ser199, Ser202 and Thr205) reduces the folding of the N-terminus into the paperclip conformation (Jeganathan et al., 2008), suggesting that phosphorylation of tau at these sites could increase PAD exposure. To determine whether AT8 phosphorylated tau monomers could inhibit anterograde FAT, we perfused AT8 pseudophosphorylated tau (Ser199Glu/Ser202Glu/Thr205Glu; AT8 tau; Figure 1B) into isolated squid axoplasms. Soluble AT8 tau monomers inhibited anterograde FAT (Figure 6A and 6C), while

retrograde transport was unaffected (Figure 6A and 6D). These data indicate that phosphorylation of tau at the AT8 epitope, which is associated with hyperphosphorylation of tau in AD and other tauopathies, renders soluble monomeric tau capable of inhibiting anterograde FAT.

A novel mutation in the *tau* gene associated with a case of FTD was recently reported (Rovelet-Lecrux et al., 2009). This tau mutation results in deletion of amino acids 144-273 encompassing the proline-rich domain and the first MTBR (Δ 144-273 tau; Figure 1C). Deletion of these tau domains should result in constitutive exposure of PAD because they play a pivotal role in the intramolecular folding of the N-terminus. Consistent with our model, Δ 144-273 tau monomers significantly inhibited anterograde FAT (Figure 6B and 6C), while retrograde FAT remained unaffected (Figure 6B and 6D). Taken together, these data indicate that disease-associated modifications and mutations in tau that increase exposure of PAD promote activation of the PP1-GSK3 pathway and inhibition of anterograde FAT.

Increased Exposure of PAD is an Early Event in AD

Results from isolated axoplasm experiments suggested a molecular basis for tau toxicity. To evaluate the relevance of these findings in human disease, we generated a novel monoclonal antibody termed “Tau N-Terminal 1” or TNT1 antibody, which specifically recognizes PAD. The TNT1 antibody exhibits a very high affinity ($EC_{50}=5.5$ ng/ml) for WT tau containing PAD (Figure 7A) and does not react with tau missing PAD (Δ 2-18 tau) in ELISAs (WT tau: mean $A_{450}=2.4 \pm 0.03$; Δ 2-18 tau: mean $A_{450}=0.003 \pm 0.004$) and immunoblots (Figure 7A). Using the TNT1 antibody, we examined the availability of PAD in AD and control brains. First, we probed samples of soluble and insoluble tau isolated from the frontal cortex of control and severe AD cases using immunoblots. The

pattern of TNT1 reactivity on Western blots was consistent with that of tau isolated from human brain as indicated by the Tau12 (Horowitz et al., 2004) and R1 tau antibodies (Berry et al., 2004) (two well-characterized tau-specific antibodies), confirming the specificity of TNT1 for tau protein and not non-tau proteins in human brains (Figure 7B). Under these denaturing conditions, TNT1 reacted similarly with soluble tau from controls and AD samples, as well as with insoluble tau from AD samples (Figure 7B).

To further explore the characteristics of TNT1 reactivity, we analyzed soluble and insoluble tau fractions using dot blots, which like Western blots are solid-state assays, but unlike Western blots the proteins are not denatured. In dot blots, TNT1 showed a significantly increased selectivity for both soluble and insoluble tau isolated from AD, as opposed to control brains (Figure 7C). The lack of distinction between control and AD tau in denaturing conditions by TNT1, but significant selectivity for AD tau over control tau in non-denaturing conditions highlights that TNT1 reactivity is indicative of PAD exposure, which is dependent upon maintaining protein conformation. Thus, these results suggest that PAD is more readily available in the native state of tau derived from AD brains, as compared to tau derived from control brains, and TNT1 immunoreactivity can be used as an indicator of PAD exposure.

Immunohistochemistry with TNT1 was performed on tissue sections from controls (Braak stages I-II; n=4) and severe AD (Braak stages V-VI; n=4) cases to determine when and where PAD exposure occurs in the disease process. Qualitative evaluation of sections from the entorhinal cortex (Figure 7D) and hippocampus (Figure 7E), as well as the inferior and superior temporal gyri (data not shown) indicated that TNT1 reactivity followed the typical pattern of Braak staging (Braak and Braak, 1991). In age-matched control cases, TNT1 diffusely labeled neurons in the earliest stages of tau deposition (e.g. early pre-tangle neurons), and a subset of these neurons contained small,

darkly stained, globular intracellular inclusions (Figure 7F). A small number of neurons in control cases exhibited strong TNT1 immunoreactivity of seemingly immature tangle-like inclusions that extended into both apical and basal dendrites (Figure 7F). With progression into severe AD, there was a remarkable increase in the amount of TNT1 immunoreactive inclusions (Figure 7D and 7E), which included neurofibrillary tangles (Figure 7G), neuropil threads (Figure 7G) and neuritic plaques (Figure 7H). These data indicated that increased PAD exposure, as revealed by TNT1 immunoreactivity, occurs early in AD and remains present throughout the disease process.

We next determined the relationship between PAD exposure and AT8 phosphorylation in tau since this modification rendered soluble tau monomers toxic to FAT (see above). Tissue sections from the hippocampus of controls (Braak stages I-II; n= 4), mild AD (Braak stages III-IV; n=5) and severe AD (Braak stages V-VI; n=4) were double-stained with TNT1 and AT8 antibodies using immunofluorescence (Figure 7I-K). Qualitative observations indicated that TNT1 immunoreactivity might appear slightly before the development of AT8 phosphorylation in the course of AD. Numerous TNT1-positive structures (e.g. cell bodies and threads) in control cases (Figure 7I) did not appear to be AT8-positive. As pathology progressed into mild AD (Figure 7J), the amount of both TNT1 and AT8 increased. Finally, in severe AD stages (Figure 7K) both TNT1 and AT8 were abundant and exhibited nearly complete co-localization. It should be noted that when present, AT8 immunoreactivity was almost always co-localized with TNT1 in all cases analyzed. Taken together, these data suggest increased PAD exposure represents an early event in AD pathogenesis and that AT8 may not be required for PAD exposure *in situ*.

Discussion

Recently, we demonstrated that WT tau filaments inhibit anterograde FAT by activating a PP1-GSK3 signaling cascade through a mechanism requiring the N-terminus of tau (LaPointe et al., 2009). Extending those observations, we have identified a tau domain comprised of amino acids 2-18 that is both necessary and sufficient to activate the PP1-GSK3 cascade and inhibit anterograde FAT. These effects suggest a novel function for tau and define a new functional motif in tau, the phosphatase-activating domain (PAD). Inhibition of anterograde FAT by the 6D and 6P isoforms of tau and a synthetic PAD peptide demonstrates that this effect does not require tau binding to microtubules or tau aggregation. Our results demonstrate that exposure of PAD is a critical factor mediating the ability of pathogenic forms of tau to inhibit FAT. Consistent with this hypothesis, two disease-associated tau modifications that increase PAD exposure inhibited anterograde FAT as soluble monomers. Further supporting the role of PAD exposure as an important disease-related event, the novel PAD-specific antibody, TNT1, selectively labeled both soluble and insoluble tau isolated from AD brains, but not that from controls. Moreover, qualitative observations of TNT1 immunoreactivity in human AD tissue sections suggested that PAD exposure represents an early pathological event that closely associates in time with AT8 phosphorylation. These data have important implications regarding the role of tau in disease pathogenesis and provide a mechanism through which disease-associated modifications and/or mutations in tau lead to a toxic gain-of-function in AD and other tauopathies.

The demonstration that PAD is a region within tau that can modulate the PP1-GSK3 cascade has significant implications for tau pathology and suggests a novel biological role for tau regulating the activity of phosphotransferases and anterograde FAT (Figure 8). Tau has been shown to target PP1 to microtubules and can directly interact

with PP1 (Liao et al., 1998). Amino acids 5-8, located within PAD, comprise one of the proposed PP1 binding sites in tau (Liao et al., 1998). Additionally, tau and GSK3 β have been co-purified as part of a high molecular weight complex in association with purified microtubules (Sun et al., 2002). Thus, in addition to microtubule stabilization, tau may bind and target phosphotransferases to the vicinity of microtubules (Shahani and Brandt, 2002) and regulate FAT through modulation of these enzymes (Figure 8A), a functional scheme that may be disrupted in disease (Figure 8B).

Deficits in FAT have previously been implicated in the neuronal dysfunction associated with dysferopathies such as AD, non-AD tauopathies and other neurodegenerative diseases (Morfini et al., 2002a; Morfini et al., 2009; Roy et al., 2005). In AD brains, dystrophic neurites, synaptic loss and protein mislocalization (Dessi et al., 1997; Scheff et al., 1990) are all consistent with FAT deficits. Accordingly, studies in various animal models of AD and tauopathies have reported alterations in axonal transport (e.g. axonal swellings, synaptic loss and impaired vesicle transport) (for review see Higuchi et al., 2006). Interestingly, tau appears to play a critical role in amyloid- β -mediated axonal transport disruption, as genetically removing tau mitigates the effects of treating neurons with amyloid- β oligomers (Vossel et al., 2010). These studies suggested a link between tau, axonal dysfunction and neurodegenerative disease; however, until this report the molecular mechanisms linking deficits in FAT to disease-related modifications of tau had remained unclear (Morfini et al., 2002a).

Several studies on the role for tau in axonal transport dysfunction proposed a mechanism involving direct interference of tau with the binding of conventional kinesin to microtubules (Ebner et al., 1998; Seitz et al., 2002; Vershinin et al., 2007). However, overexpression of human tau in transgenic mice did not affect FAT rates in the optic

nerve (Yuan et al., 2008) and levels of soluble WT tau 20 fold greater than endogenous levels of tau had no effect on FAT in squid axoplasm (Morfini et al., 2007). Studies on filamentous tau (LaPointe et al., 2009) and the current data provide evidence for an alternative mechanism independent of microtubule binding that does not require aggregation. Specifically, many of the tau species examined in these studies do not effectively bind microtubules (e.g. tau aggregates, 6D/6P tau, PAD peptide and FTD Δ 144-273 tau), and many were used as monomers (e.g. 6D/6P tau, PAD peptide, FTD Δ 144-273 tau, and AT8 tau). Moreover, we have established that tau inhibits anterograde FAT by activating the PP1-GSK3 cascade with nearly all of the tau species tested thus far. Together, these data clearly demonstrate that tau-mediated disruption of anterograde FAT via the PP1-GSK3 cascade is independent of microtubule binding and does not require aggregation.

Based on our prior studies (LaPointe et al., 2009), it was unclear why monomeric WT tau failed to affect FAT even though the PAD motif was present. Tau was originally thought to exist in an extended random coil state based on spectroscopic studies (Mandelkow et al., 2007). However, fluorescence resonance energy transfer studies indicated that soluble monomeric WT tau adopts a folded paperclip conformation (Jeganathan et al., 2008; Jeganathan et al., 2006), that may prevent PAD from activating the PP1-GSK3 cascade. Conversely, conformations of tau that increase exposure of PAD should promote inhibition of anterograde FAT.

We tested this hypothesis using numerous forms of tau in which the accessibility of PAD is increased. In tau aggregates, both termini of tau are believed to maintain a random coil structure extending from the filament core formed by the MTBRs (Barghorn et al., 2004). Evidence also suggests filamentous tau favors an Alz50-type conformation (Carmel et al., 1996), in which the N-terminus is in close contact with the MTBRs.

However, the results presented here suggest that the *extreme* N-terminus of tau remains accessible in filamentous tau. The 6D and 6P isoforms of tau lack the MTBRs and C-terminus (Luo et al., 2004) necessary for the paperclip conformation, leaving PAD constitutively available, and the PAD peptide is composed of only amino acids 2-18. Similarly, a recombinant pseudophosphorylated AT8 tau exhibits reduced folding of the N-terminus into the paperclip conformation (Jeganathan et al., 2008). Finally, deletion of amino acids 144-273 in the FTD tau construct should dramatically reduce or eliminate the ability of the N-terminus to fold into the paperclip, as this deletion removes the proline-rich hinge region involved in N-terminal folding. Consistent with our hypothesis, all of these “PAD-exposed” tau species inhibited anterograde FAT; thus, exposure of PAD provides a common mechanism of toxicity for biochemically heterogeneous forms of pathogenic tau.

Highlighting the relevance of results obtained from studies in isolated squid axoplasm, a PAD-specific antibody (TNT1) that preferentially recognizes disease-related forms of tau confirmed the importance of PAD exposure in human disease pathogenesis. Data obtained from immunostaining studies in human brain tissue indicated that increased PAD exposure occurs very early in the process of tau inclusion formation and AT8 is closely associated with PAD exposure during AD progression. Importantly, quantitative analyses are required to confirm our observations and conclusively determine the time course of PAD exposure in relation to other tau modifications during the progression of disease in humans. However, in combination with our squid axoplasm data, it is reasonable to assume that AT8 modification abundantly found in AD (Braak et al., 1994) and other tauopathies (Sakai et al., 2006; Shiarli et al., 2006; Takahashi et al., 2002) may facilitate PAD exposure and tau-mediated FAT dysfunction *in situ*. Additionally, our data suggest a cyclical relationship between PAD exposure and AT8

phosphorylation in which the AT8 modification might result, at least in part, from the increased GSK3 activation triggered by abnormally exposed PAD since S199, S202 and T205 are GSK3 β phosphorylation sites.

Since neurons are thought to maintain function for years while bearing tau inclusions (Morsch et al., 1999) it is reasonable to assume the existence of compensatory mechanisms that render pathogenic forms of tau innocuous. Phosphorylation of tyrosine 18 (within PAD) by kinases such as fyn (Lee et al., 2004) or N-terminal cleavage of tau (Horowitz et al., 2004; Sengupta et al., 2006) may represent such protective mechanisms. Jeganathan and coworkers demonstrated that combinations of phosphorylation events have different effects on tau folding (Jeganathan et al., 2008). While AT8 alone reduces N-terminal folding, AT8 in combination with the AT100 (T212/S214) and PHF-1 (S396/S404) phosphorylation epitopes causes tau to fold into a more compact paperclip conformation. Regulation of tau conformation through protein-protein or intraprotein interactions as well as through modifications like phosphorylation or proteolysis might be part of a complex set of events that regulate tau function, including microtubule binding, modulation of phosphotransferase-based signaling pathways and anterograde FAT.

Although tau has been recognized as a potential therapeutic target in AD and tauopathies, choosing therapeutic targets and testable outcome measures has proven difficult without an understanding of mechanisms through which tau can influence disease progression. The identification of PAD and its effects on phosphotransferase activity and FAT regulation allow us to propose specific targets for intervention. For instance, reducing the activities of PP1 and/or GSK3 may reduce the toxic potential of pathogenic forms of tau. Alternatively, therapeutic strategies aimed at directly blocking PAD exposure and preventing it from activating the PP1-GSK3 cascade may provide yet another viable point of intervention.

Figure Legends

Figure 1. Schematic diagram of the tau constructs used. **A**, Wild-type ht40 (WT tau) is the largest isoform of tau found in the adult CNS. WT tau contains the PAD motif corresponding to amino acids 2-18 (black box), two alternatively spliced N-terminal exons (E2 and E3), and four microtubule-binding repeats (MTBRs 1-4). **B**, The AT8 tau protein was pseudophosphorylated at S199, S202 and T205 by mutagenesis of S→E and T→E in order to mimic the phosphoepitope recognized by the AT8 antibody (Biernat et al., 1992; Goedert et al., 1995) in hyperphosphorylated tau. **C**, The Δ 144-273 tau mutant protein corresponds to a deletion of amino acids 144-273 (the proline-rich region and MTBR1) that was found in a patient with an autosomal dominant familial form of FTD (Rovelet-Lecrux et al., 2009). **D**, The 6D tau protein is a non-canonical N-terminal isoform containing amino acids 1-143 with an additional 11 unique C-terminal amino acids (Luo et al., 2004). **E**, The 6P protein is the same as 6D, except the last 11 C-terminal amino acids are different (Luo et al., 2004). **F**, The Δ 2-18 6D protein has amino acids 2-18 deleted from the N-terminus. **G**, Synthetic peptide composed of the PAD sequence (amino acids 2-18 from WT tau). **H**, Synthetic peptide composed of the same amino acids as the PAD peptide, but rearranged in a random order.

Figure 2. N-terminal tau isoforms, 6D and 6P tau, selectively inhibits anterograde FAT. Vesicle motility assays in isolated squid axoplasm. Individual velocity ($\mu\text{m}/\text{sec}$) rate measurements (arrowheads) are plotted as a function of time (minutes). Black arrowheads and lines represent anterograde FAT rates (conventional kinesin-dependent) and grey arrows and lines represent retrograde FAT rates (cytoplasmic dynein-dependent). **A**, Perfusion with soluble wild-type tau monomers (WT tau) did not affect FAT rates in the anterograde (\blacktriangleright) or retrograde (\blacktriangleleft) direction. **B**, In contrast, soluble 6D tau monomers,

which cannot bind microtubules, selectively inhibited the rate of anterograde FAT, while retrograde FAT was not significantly affected. **C**, Quantitative analysis of FAT demonstrates that 6D and 6P (n=5) tau caused a significant reduction in anterograde FAT when compared to WT tau monomers (unpaired t-test; *p<0.01). **D**, Retrograde FAT was unaffected by 6D and 6P tau.

Figure 3. 6D tau inhibits anterograde FAT via activation of a PP1-GSK3 cascade. **A**, Co-perfusion of 6D tau and I-2, a specific PP1 inhibitor, prevented inhibition of anterograde (▶) FAT elicited by 6D (compare with Figure 2B). **B**, Co-perfusion of 6D tau and ING-135, a specific GSK3 inhibitor, similarly prevented inhibition of anterograde FAT. **C**, Quantitative analysis of FAT shows that inhibition of PP1 (via I-2) or inhibition of GSK3 (via ING-135) completely prevents the anterograde FAT inhibition elicited by 6D tau (one-way ANOVA; *p<0.01 versus WT Tau, 6D+I-2, 6D+ING-135). **D**, Retrograde FAT (◀ retrograde) was unaffected by 6D, regardless of whether inhibitors were present.

Figure 4. PAD is necessary and sufficient to trigger anterograde FAT inhibition. **A**, Perfusion of Δ 2-18 6D into isolated axoplasm did not affect FAT in either direction (▶ anterograde; ◀ retrograde). **B**, In contrast, perfusion of the PAD peptide, which corresponds to amino acids 2-18 in WT tau, caused a reduction of anterograde, but not retrograde FAT rates. **C**, A scrambled version of the PAD peptide was used as a control, and when applied to squid axoplasms did not affect FAT. **D**, Quantitative analysis of FAT rates indicates that PAD peptide significantly inhibits anterograde FAT (one-way ANOVA; *p<0.01), while deletion of the PAD sequence in 6D (Δ 2-18 6D) and scrambling the PAD peptide (Scram PAD) completely prevented inhibition of anterograde FAT. **E**, Retrograde FAT rates were unaffected in all groups tested.

Figure 5. PAD peptide inhibits anterograde FAT by activation of the PP1-GSK3 cascade. **A**, Co-perfusion of PAD peptide with I-2, a specific PP1 inhibitor, had no effect on anterograde (▶) or retrograde (◀) FAT in squid axoplasm (compare with Figure 4B). **B**, Co-perfusion of PAD peptide with ING-135, a specific GSK3 inhibitor, similarly prevented inhibition of anterograde FAT by PAD. **C**, Quantitative analysis of FAT reveals that PAD peptide alone specifically and significantly inhibits anterograde FAT (one-way ANOVA; * $p > 0.01$), while addition of either PP1 or GSK3 inhibitors completely prevented the reduction in anterograde FAT rates induced by the PAD peptide. **D**, Retrograde FAT rates were unaffected in all groups tested. **E**, P^{32} -c-Jun (a phosphatase substrate) undergoes dephosphorylation by endogenous axoplasmic phosphatases after 30 minutes. Axoplasms were perfused with ^{32}P -c-Jun alone (Ctrl) or with ^{32}P -c-Jun and either okadaic acid (OK, 1 μ M) or Inhibitor-2 (I-2, 400nM). Aliquots were taken immediately after perfusion (0') and 30 minutes later (30'), and analyzed by autoradiography. **F**, ^{32}P -c-Jun was dephosphorylated to a greater extent in PAD-perfused axoplasms than its scrambled PAD-perfused "sister" counterpart, suggesting increased activation of endogenous axoplasmic phosphatases by PAD. "Sister" axoplasms from the same squid were perfused with ^{32}P -c-Jun and either scrambled PAD (Scr) or PAD peptides and analyzed by autoradiography (n=2 sets of sister axoplasms).

Figure 6. Tau monomers with disease-associated modifications inhibit anterograde FAT. **A**, Perfusion of squid axoplasm with soluble tau monomers containing mutations S199E, S202E and T205E (phosphomimicking the AT8 epitope seen in hyperphosphorylated tau; AT8 tau) causes a reduction in anterograde (▶), but not retrograde (◀) FAT. **B**, Similarly, perfusion of squid axoplasm with soluble Δ 144-273 tau monomer (mimicking

a deletion mutation associated with a case of FTD), results in a reduction in anterograde, but not retrograde FAT. **C**, Quantitative analysis of FAT rates demonstrates that AT8 tau monomers and $\Delta 144-273$ tau monomers significantly inhibit anterograde FAT, when compared to WT tau monomers (one-way ANOVA; * $p < 0.01$). **D**, Retrograde FAT rates remained unaffected for all pathogenic tau monomers tested.

Figure 7. PAD immunoreactivity in AD brains. **A**, An ELISA titer of the TNT1 antibody using recombinant wild-type (WT) tau indicates a LogEC_{50} of 5.25 or a 1:180,000 dilution of a 1mg/ml stock (final concentration 5.5 ng/ml). TNT1 does not react with tau lacking PAD (amino acids 2-18; $\Delta 2-18$) in immunoblots. **B**, TNT1 labels enriched soluble tau from the frontal cortex of both control (Con Sol) and AD brains (AD Sol) as well as purified paired helical filament tau (AD PHF) from AD frontal cortex in *denaturing conditions* on Western blots. Comparison of TNT1 blots with Tau12 and R1 tau blots clearly indicates that TNT1 recognizes tau proteins and not other proteins in human brains. **C**, Dot blots of TNT1 under *non-denaturing conditions*. TNT1 preferentially labels soluble and PHF tau isolated from AD brains as opposed to tau isolated from control brains indicating that PAD-exposed tau conformations are more abundant in AD. Quantification of both soluble and insoluble tau fractions is depicted as TNT1:Tau12 optical density (OD) ratios in the bar graph; Tau12 was used to measure total tau. Unpaired t-test, * $p \leq 0.05$. **D and E**, TNT1 immunohistochemistry in the entorhinal cortex (**D**) and hippocampus (CA1 region; **E**) of human Braak stages I-II (control) or V-VI (severe AD) cases. Spatial and temporal patterns of TNT1 staining followed those of Braak staging, starting in the entorhinal cortex (**D**) and then appearing in the hippocampus (**E**) before involving temporal cortical gyri in later stages (data not

shown). Scale bars are 100 μ m. **F**, TNT1 staining in Braak stage I-II cases. TNT1 diffusely labeled the cytoplasm of “pre-tangle” CA2 neurons in the very early stages of inclusion formation (\rightarrow). A subset of diffusely stained neurons contained globular cytoplasmic inclusions (\rightarrow). A few neurons contained strongly labeled seemingly less mature tau inclusions that extended into the basal (\wedge) and apical (\blacktriangle) dendrites. **G and H**, TNT1 in Braak stages V-VI; note that large amounts of the classical tau inclusions, neurofibrillary tangles (\rightarrow ; **G**), neuropil threads (\blacktriangle ; **G**) and neuritic plaques (*; **H**), were present in the regions analyzed (CA1 shown). Scale bars are 20 μ m. **I-K**, Representative images of TNT1 (red) and AT8 (green) double-label immunofluorescence in the hippocampus (CA1/CA2 region). **I**, The TNT1 epitope appears to precede that of AT8 in controls cases (Braak stages I-II). **J**, Both TNT1 and AT8 continue to accumulate in mild AD (Braak stages III-IV). **K**, TNT1 and AT8 appear with nearly complete coincidence in severe AD (Braak stages V-VI). Note that when present, AT8 extensively co-localizes with TNT1 at all Braak stages. Scale bars are 50 μ m.

Figure 8. Proposed role of PAD in anterograde fast axonal transport regulation and in axonal transport dysfunction in disease. **A**, Tau is normally localized to microtubules where, in addition to stabilizing microtubules, it may regulate local cargo delivery through PAD-mediated activation of the PP1-GSK3 cascade. When tau is folded into the paperclip conformation, membrane-bound cargoes are transported by conventional kinesin along microtubules because PAD is hidden. In contrast, cargo is delivered at appropriate locations where tau is in an extended conformation and PAD is exposed. The exposure of PAD activates PP1-GSK3, leading to phosphorylation of kinesin light chains and release of cargoes. **B**, In disease states, tau aggregations and/or modifications expose PAD in tau that no longer binds to microtubules, promoting aberrant activation of the

PP1-GSK3 cascade, increased inhibition of anterograde FAT and ultimately neuron dysfunction/degeneration. Abnormal presentation of the biologically active PAD motif represents a critical factor in determining whether disease-associated forms of tau inhibit anterograde FAT.

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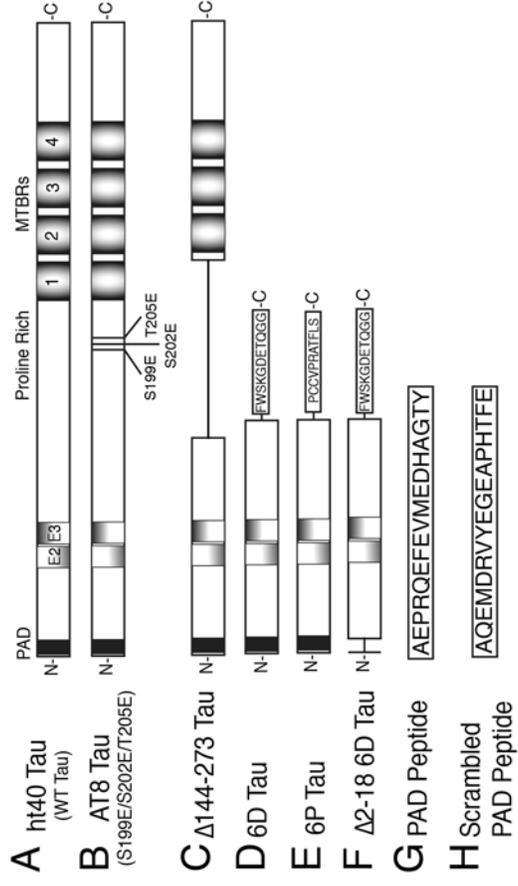
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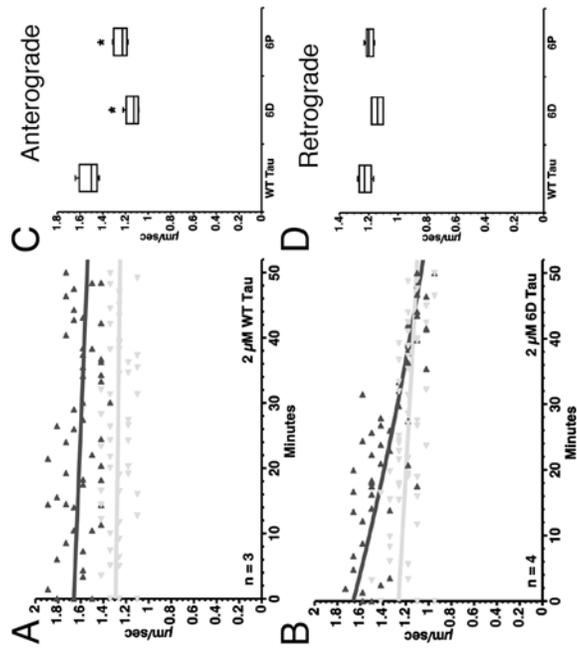
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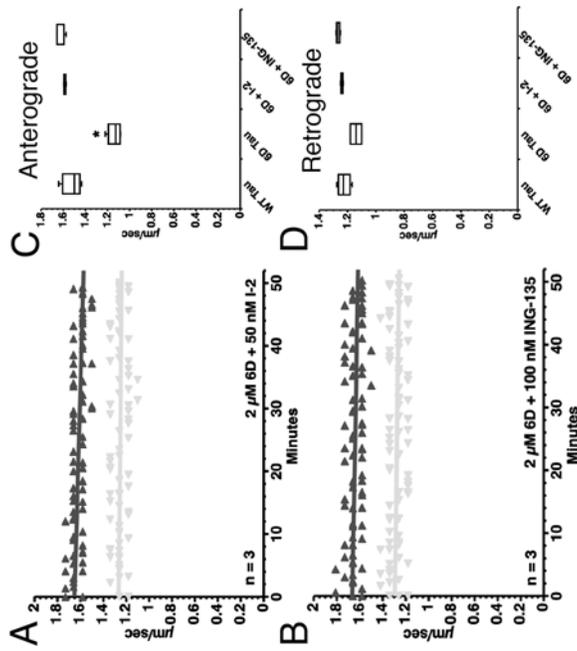
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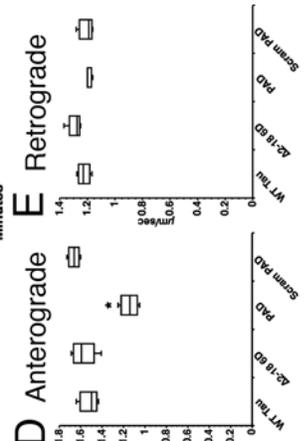
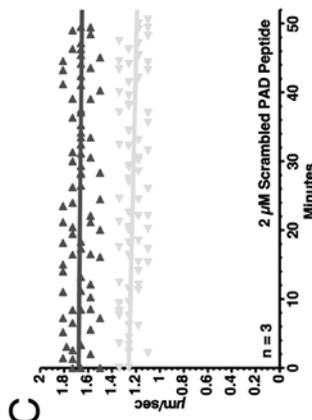
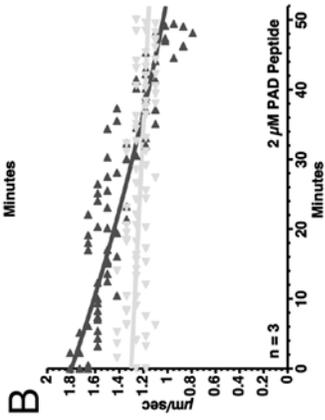
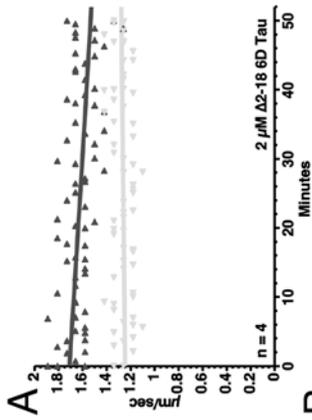
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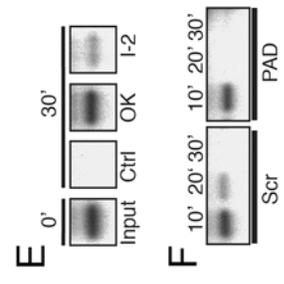
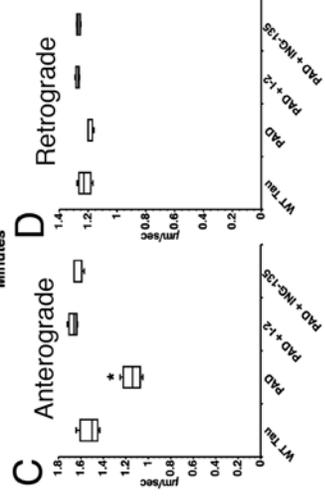
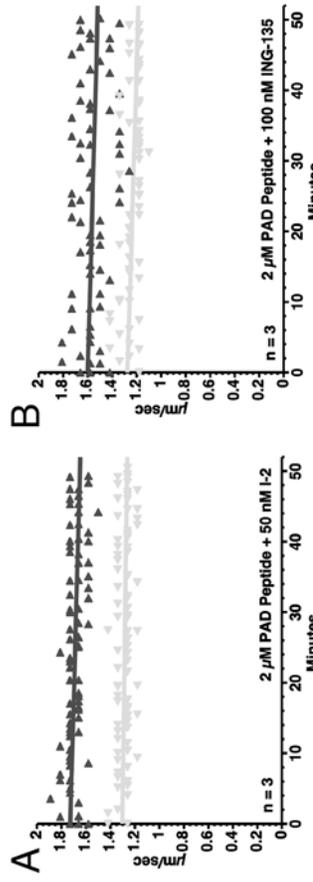
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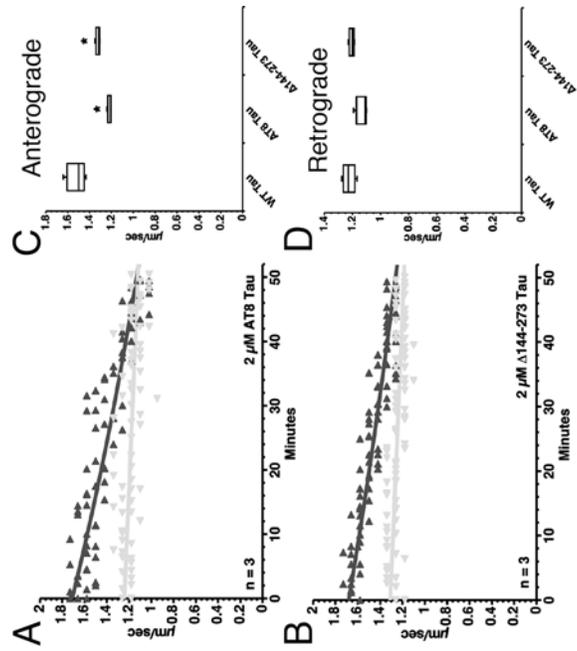












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