Neurobiology of Disease

Soluble Conformers of Aβ and Tau Alter Selective Proteins Governing Axonal Transport

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Despite the demonstration that amyloid-β (Aβ) can trigger increased tau phosphorylation and neurofibrillary tangle (NFT) formation *in vivo*, the molecular link associating Aβ and tau pathologies remains ill defined. Here, we observed that exposure of cultured primary neurons to Aβ trimers isolated from brain tissue of subjects with Alzheimer's disease led to a specific conformational change of tau detected by the antibody Alz50. A similar association was supported by postmortem human brain analyses. To study the role of Aβ trimers *in vivo*, we created a novel bigenic Tg-Aβ+Tau mouse line by crossing Tg2576 (Tg-Aβ) and rTg4510 (Tg-Tau) mice. Before neurodegeneration and amyloidosis, apparent Aβ trimers were increased by ~2-fold in 3-month-old Tg-Aβ and Tg-Aβ+Tau mice compared with younger mice, whereas soluble monomeric Aβ levels were unchanged. Under these conditions, the expression of soluble Alz50-tau conformers rose by ~2.2-fold in the forebrains of Tg-Aβ+Tau mice compared with nontransgenic littermates. In parallel, APP accumulated intracellularly, suggestive of a putative dysfunction of anterograde axonal transport. We found that the protein abundance of the kinesin-1 light chain (KLC1) was reduced selectively *in vivo* and *in vitro* when soluble Aβ trimers/Alz50-tau were present. Importantly, the reduction in KLC1 was prevented by the intraneuronal delivery of Alz50 antibodies. Collectively, our findings reveal that specific soluble conformers of Aβ and tau cooperatively disrupt axonal transport independently from plaques and tangles. Finally, these results suggest that not all endogenous Aβ oligomers trigger the same deleterious changes and that the role of each assembly should be considered separately.

Key words: Alzheimer's disease; amyloid-beta; axonal transport; brain; oligomer; tau

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Significance Statement

The mechanistic link between amyloid-β (Aβ) and tau, the two major proteins composing the neuropathological lesions detected in brain tissue of Alzheimer's disease subjects, remains unclear. Here, we report that the trimeric Aβ species induce a pathological modification of tau in cultured neurons and in bigenic mice expressing Aβ and human tau. This linkage was also observed in postmortem brain tissue from subjects with mild cognitive impairment, when Aβ trimers are abundant. Further, this modification of tau was associated with the intracellular accumulation of the precursor protein of Aβ, APP, as a result of the selective decrease in kinesin light chain 1 expression. Our findings suggest that Aβ trimers might cause axonal transport deficits in AD.

Introduction

In our current understanding of the physiopathology of Alzheimer's disease (AD), the soluble forms of the amyloid-β peptide

(Aβ) and the microtubule-associated protein tau have been proposed to be more important than the fibrillar aggregates that have classically characterized this disorder (Walsh et al., 2002; Cleary et al., 2005; Santacruz et al., 2005; Lesné et al., 2006; Berger et al., 2007; Roberson et al., 2007; Shankar et al., 2008). Despite the seminal demonstrations that Aβ exposure can lead to increased

tau phosphorylation and neurofibrillary tangle (NFT) formation in animals (Götz et al., 2001; Lewis et al., 2001; Oddo et al., 2003), the exact molecular mechanisms associating Aβ and tau remain poorly understood (Attems et al., 2011; Larson and Lesné, 2012; Lesné, 2013).

Due to the inherent biology of neuronal cells, axonal transport is critical for neuronal function and survival. Multiple neurodegenerative disorders, including AD, present with alterations of fast axonal transport, which have been proposed to represent an early pathological event (Goldstein, 2001; Stokin et al., 2005; Ittner et al., 2009; Morfini et al., 2009; Muresan and Muresan, 2009). Soluble assemblies of Aβ, also called Aβ oligomers (oAβs), have been shown to be capable of inhibiting axonal transport in cultured cells (Rui et al., 2006; Pigino et al., 2009). Additional reports refined this concept by demonstrating that oligomeric mixtures of synthetic Aβ disrupt axonal transport *in vitro* (Decker et al., 2010; Vossel et al., 2010; Vossel et al., 2015).

In addition to Aβ, tau is known to be concentrated preferentially in axons, where it stabilizes microtubules that serve as tracks for the transport of organelles, vesicles, and proteins (Hirokawa and Takemura, 2005) and has been proposed to induce neuronal cell death by interfering with microtubule-dependent axonal transport (Stamer et al., 2002). Despite convincing observations showing that tau alters axonal transport *in vitro* (Ebneth et al., 1998; Dixit et al., 2008), it is less clear whether tau acts similarly *in vivo* (Yuan et al., 2008). Recent studies indicated that, although tau did not appear to affect axonal transport under baseline conditions, tau protein levels were critical for axonal transport in the presence of synthetic Aβ oligomers (Vossel et al., 2010).

While assessing the effects of purified forms of endogenous oAβs on tau posttranslational modifications, we found that AD-brain-derived Aβ trimers applied onto primary neurons at single-digit nanomolar concentrations induced a selective conformation change of tau detected by the antibody Alz50 (Carmel et al., 1996). Supporting this *in vitro* finding, we found that protein levels of Aβ trimers, described previously to peak in the brain tissues of Religious Orders Study (ROS) participants with mild cognitive impairment (MCI) (Lesné et al., 2013), were positively correlated with soluble Alz50-tau levels. Upon characterizing the newly created bigenic Tg-Aβ+Tau mouse model overexpressing the human APP and human tau, we observed that soluble Aβ trimers increased independently of monomeric Aβ levels before neurodegeneration and amyloidosis in the forebrains of these mice. In association with the rise in Aβ trimers observed in young bigenic mice, soluble Alz50-positive tau levels were also elevated, whereas other pathological forms of tau were not. In parallel, APP accumulated intracellularly in brain tissue of bigenic mice, suggesting possible axonal transport defects. When analyzing putative modulations in the abundance of proteins governing axonal transport, the protein expression of the light chain of kinesin-1 (KLC1) was lowered markedly, whereas other motor proteins appeared to be unaffected. To evaluate the potential effects of Aβ trimers on proteins regulating axonal transport, we exposed primary cultured neurons to purified Aβ species. These conditions recapitulated the selective changes in KLC1 observed *in vivo*, indicating that Aβ trimers are a potent disruptor of KLC1 expression in neurons. Finally, we showed that this effect was not dependent on the expression of the cellular form of the prion protein PrP^C, but did require the presence of tau gene expression and Alz50-tau conformers.

Materials and Methods

Transgenic mice
Mice from the APP line Tg2576 (MGI_2385631), which express the human APP with the Swedish mutation (APP$^{KM670/671NL}$) directed by the hamster prion protein promoter (Hsiao et al., 1996), were crossed with rTg4510 mice (MGI_4819866) (Ramsden et al., 2005; Santacruz et al., 2005) overexpressing the P301L mutant of four-repeat tau lacking the N-terminal sequences 4R0N (tau^{P301L}). Both lines were kindly provided by Dr. Karen Ashe (University of Minnesota). Briefly, Tg4510 responders were mated with CKTTA activators (129S6) to generate FVB129S6F1-rTg4510 mice. rTg4510$^{+/+}$ mice were subsequently mated with Tg2576 (129S6) to generate mixed-background mice (129S6)FVB129S6F1-rTg4510xTg2576$^{+/+/+}$. Dr. Adriano Aguzzi (University Hospital of Zurich, Switzerland) kindly provided *Prnp*-null mice (MGI_1888773) (Büeler et al., 1992). Htau (MGI_3057129) (Andorfer et al., 2003) and *Mapt*-null mice (Tucker et al., 2001) were purchased from Jackson Laboratories. All experiments described here were conducted in full accordance with Association for Assessment and Accreditation of Lab Animal Care and Institutional Animal Care and Use Committee guidelines, with every effort made to minimize the number of mice used. Both male and female mice were used in all experiments. Experimenters were kept blinded to the genotype of the animal groups until raw data were obtained.

Primary cell cultures
Mouse cortical cultures of neurons were prepared from 14- to 15 d-old embryos as described previously (Lesné et al., 2005; Larson et al., 2012) using 5×10^5 cells/dish. After 3 d *in vitro* (DIV), neurons were treated with 10 μM cytosine β-D-arabinofuranoside (AraC) to inhibit proliferation of non-neuronal cells. All experiments were performed on nearly pure neuronal cultures (>98% of microtubule associated protein-2 immunoreactive cells) after 12–14 DIV. Six to eight 35 mm dishes per culture per condition were used across three independent experiments.

Protein extractions
For analyzing Aβ species, two extractions protocols described previously were used (Lesné et al., 2006; Shankar et al., 2008; Sherman and Lesné, 2011). In particular, membrane-enriched protein extracts (MB extracts) refer to protein lysates obtained after the third step of a serial extraction with a lysis RIPA buffer comprised of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 3% SDS, and 1% deoxycholate. As detailed in a methodology chapter published recently (Sherman and Lesné, 2011), samples were then centrifuged at $16,100 \times g$ for 90 min. Supernatants were collected and pellets further extracted with formic acid to analyze fibrillar/deposited proteins. It is possible that the use of the RIPA lysis buffer might strip loosely bound Aβ from plaques.

Protein amounts were determined by the Bradford protein assay (BCA Protein Assay, Pierce). All supernatants were ultracentrifuged for 60 min at $100,000 \times g$. Finally, before analysis, fractions with endogenous immunoglobulins were depleted by incubating extracts sequentially for 1 h at room temperature with 50 μl of Protein A-Sepharose, Fast Flow followed by 50 μl of Protein G-Sepharose, Fast Flow (GE Healthcare Life Sciences).

Purification of human Aβ oligomers from AD brain tissue
Human brain tissue came from participants in the ROS who died with MCI. Details of the study have been described previously (Bennett et al., 2012). The study was approved by the Institutional Review Board or Rush University Medical Center and all subjects signed informed consent and an Anatomic Gift Act. Soluble oligomeric Aβ species were purified from AD brain tissue, as reported previously by our group (Larson et al., 2012). Relative amounts of purified oligomeric Aβ were calculated based on synthetic Aβ$_{1-42}$ standards (0.001, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, and 2.5 ng; Sigma-Aldrich) run alongside the samples used for experiments.

Western blotting and quantification
SDS-PAGE. Electrophoreses were done on precast 10–20% polyacrylamide Tris-Tricine gels and 10.5–14% and 4–10.5% Tris-HCl gels (Bio-Rad). Protein levels were normalized to 2–100 μg of protein per sample
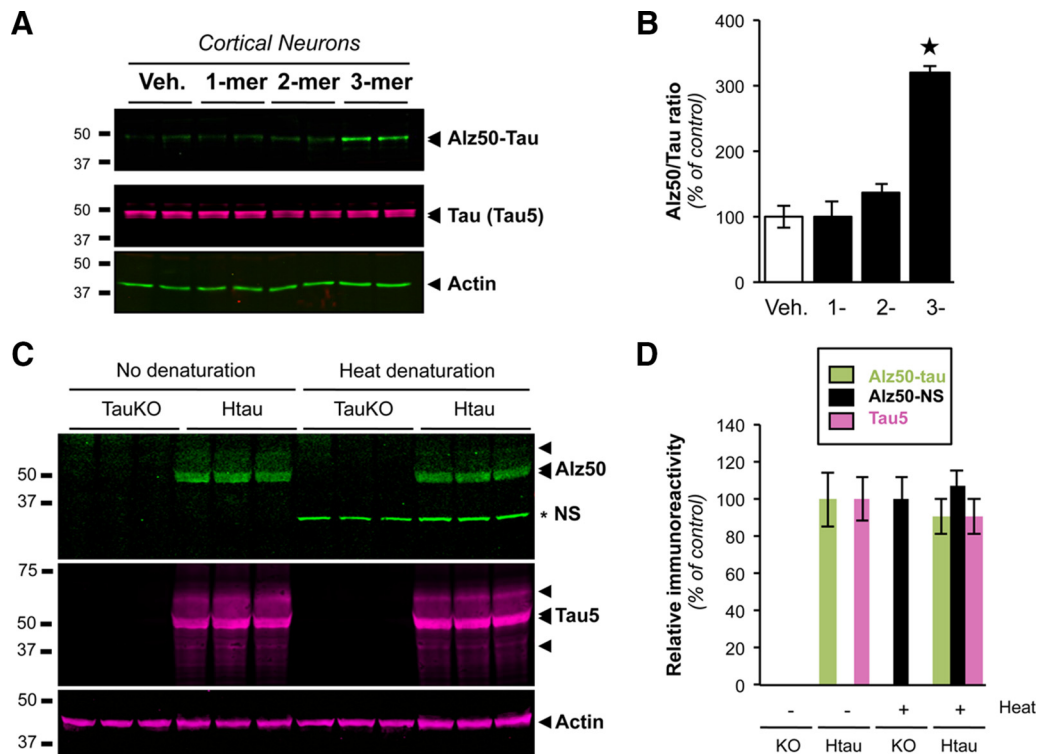
Figure 1. Application of soluble Aβ species purified from AD brain tissues induces selective tau pathological changes in mouse primary cortical neurons. *A*, Protein levels of tau conformers detected with Alz50 (Alz50-Tau) and total tau as assessed by Western blot using Alz50 and Tau5 antibodies in wild-type primary neurons exposed to human-brain-purified oligomeric Aβ (1 nM for 1 h). Actin was used as an internal control. *B*, Quantitation of the modified tau/total tau ratios across conditions revealed a significant conformational change of tau after exposure to Aβ trimers (lanes 7 and 8). This effect was not detected in neurons treated with soluble Aβ monomers or dimers. Error bars indicate mean ± SD, ANOVA ($F_{(3, 25)} = 281.604, p < 0.0001$) followed by Student's t test, ★$p < 0.05, n = 6$–8/treatment). *C, D*, Influence of sample denaturation on Alz50 immunoreactivity using Western blotting (*C, D*). Alz50-NS corresponds to the nonspecific ~25 kDa band detected by Alz50. *D*, After quantitation of the Western blot data, two-way ANOVA ($F_{(3, 23)} = 57.2621, p < 0.0001$) revealed a significant effect of the genotype ($F_{(1, 23)} = 170.766, p < 0.0001$), but no effect of the treatment ($F_{(1, 23)} = 1.017, p = 0.3427$) and no genotype*treatment interaction ($F_{(1, 23)} = 0.002, p = 0.961$). Error bars indicate the mean ± SD; $n = 6$/genotype/condition.

(depending on targeted protein) and resuspended with 4× Tricine loading buffer before boiling (5 min at 95°C with agitation at 1250 rpm).

Transfer. Thereafter, proteins were transferred to 0.2 μm nitrocellulose membrane (Bio-Rad).

Blotting. Nitrocellulose membranes were boiled in 50 ml of PBS by microwaving for 25 and 15 s with a 3 min interval. Membranes were blocked in TTBS (Tris-buffered saline plus 0.1% Tween 20) containing 5% bovine serum albumin (BSA) (Sigma-Aldrich) for 1–2 h at room temperature, and probed with appropriate antisera/antibodies diluted in 5% BSA-TTBS. Primary antibodies were probed either with anti-IgG immunoglobulins conjugated with biotin or InfraRed dyes (Li-Cor Biosciences). When biotin-conjugated secondary antibodies were used, IR-conjugated Neutravidin (Pierce) was added to amplify the signal. Blots were revealed on an Odyssey platform (Li-Cor Biosciences).

Stripping. When required, membranes were stripped using Restore Plus Stripping buffer (Pierce) for 30–180 min at room temperature depending on antibody affinity.

Quantification. Densitometry analyses were performed using Opti-Quant (Packard Bioscience) or Odyssey (Li-Cor) software. Each protein of interest was probed in three individual experiments under the same conditions and quantified by software analysis after determination of experimental conditions ascertaining linearity in the detection of the signal and are expressed as density light units (DLUs). The method used allows for a dynamic range of ~100-fold above background (0.01 × 10^6 DLUs). Respective averages were then determined across the triplicate Western blots. Normalization was performed against the neuron-specific nuclear protein NeuN, also performed in triplicate. None of the protein brain levels measured correlated with postmortem interval, arguing against potential protein degradation within human tissues (data not shown).

Immunoprecipitations

Aliquots (200 μg) of protein extracts were diluted to 1 ml with dilution buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) and incubated with the appropriate antibodies (5 μg of 6E10 or Mab2.1.3/13.1.1 antibodies) overnight at 4°C. Then, 50 μl of Protein G-Sepharose, Fast Flow (GE Life Sciences) 1:1 (v:v) slurry solution with dilution buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, pH 7.4) was added for 2 h. The beads were washed twice in 1 ml of dilution buffer and proteins eluted in 25 μl of loading SDS-PAGE buffer by boiling.

Antibodies

The following primary antibodies were used in this study: 6E10 (1:2500; BioLegend catalog #803003, RRID: AB_10175145) and 4G8 (1:2500; Bio Legend catalog #800703, RRID: AB_662812), biotinylated-6E10 (1:2500; Bio Legend catalog #803009, RRID: AB_10175146), 22C11 (1:2000; Millipore catalog #MAB348, RRID:AB_94882), Tau5 (1:2000; BioLegend catalog #806403, RRID: AB_10175718), Alz50 (1:2000; kind gifts from Peter Davies, Albert Einstein College of Medicine), 40-/42-end specific Mab2.1.3 and Mab13.1.1 (1:1000; kind gifts from Pritam Das, Mayo Jacksonville), anti-NeuN (1:5000; Millipore catalog #MAB377B, RRID:AB_177621), anti-MAP2 (1:500; Novus catalog #NB300–213, RRID:AB_2138178), anti-KLC-1 (1:1000; Millipore catalog #MAB1616, RRID:AB_94286 and Santa Cruz Biotechnology catalog #sc-25735, RRID:AB_2280879), anti-kinesin superfamily protein 5 (anti-KIF-5) (1:1000; Abcam catalog #ab62104, RRID:AB_2249625), anti-JNK-interacting protein 1 (anti-JIP-1) (1:1000; Abcam catalog #ab24449, RRID:AB_448056 and Abcam catalog #ab78948, RRID:AB_1640605), anti-Dynein (1:2000; Abcam catalog #ab75214, RRID:AB_1280872), anti-EB-3 (1:2000; Abcam catalog #ab99287, RRID:AB_10676513), anti-Actin (1:10,000; Millipore catalog #MAB1501, RRID:AB_2223041 and Sigma-Aldrich catalog #A2066, RRID:
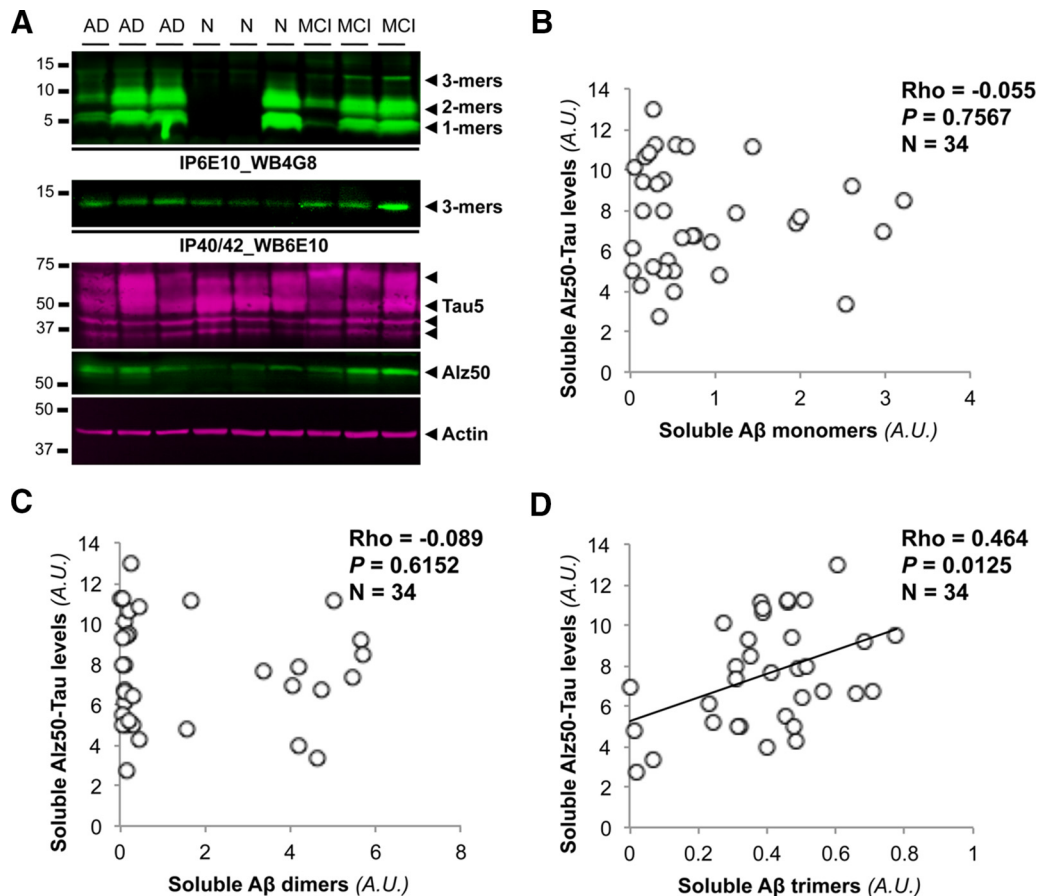
Figure 2. Soluble Aβ trimers levels are positively correlated to soluble Alz50-tau conformers in the inferior temporal gyrus of individuals diagnosed with MCI in the ROS cohort. ***A***, Representative Western blot images for Aβ and tau proteins in a subset of ROS study participants (9 out of 84). Aβ levels were measured following immunoprecipitation using 6E10 (top insert) or a mixture of 40- and 42-end specific antibodies (middle insert). 4G8, 6E10, Tau5, and Alz50 antibodies were used for Western blotting detection. Actin was used as control. ***B***–***D***, Regression analyses between low-molecular-weight soluble Aβ species and soluble Alz50-tau conformers in ROS MCI measured (Spearman's rho correlation, *n* = 34). The arbitrary units for Aβ and tau protein levels are on the same scale and correspond to $\times 10^6$ densitometry light units, as reported in Lesné et al. (2013).

AB_476693), and anti-α-tubulin (1:100,000; Sigma-Aldrich catalog #T6074, RRID:AB_477582).

Statistical analyses

When variables were non-normally distributed, nonparametric statistics were used (Spearman's rho correlation coefficients, Kruskal–Wallis nonparametric ANOVA followed by Bonferroni-corrected two-group *post hoc* Mann–Whitney U tests). When variables were normally distributed, the following parametric statistics were used (one/two-way ANOVA followed by Bonferroni-corrected two-group *post hoc* Student's *t* tests). Sample size was determined by power analysis to be able to detect statistically significant changes within a 20% variation of measured responses. Analyses were performed using JMP 11 or JMP12 (SAS Institute).

Results

Endogenous Aβ trimers induce distinct tau pathological changes *in vitro*

We reported previously that Aβ dimers and trimers purified from AD brain tissue applied at low nanomolar concentrations induce the hyperphosphorylation of tau at tyrosine 18 (Y18) mediated by the Src kinase Fyn in mouse cortical neurons (Larson et al., 2012). To determine whether other disease-relevant tau modifications were triggered under these conditions by these low-molecular-weight Aβ oligomers, we used a panel of well characterized antibodies, including CP13, PHF1, PG5, and Alz50, that detect either phosphorylation at serine residues 202 (S202),

396/404 (S396/404), and 409 (S409) or aberrant misfolding of tau, respectively (Fig. 1) Although tau was hyperphosphorylated at Y18 by purified Aβ dimers and trimers, no apparent enhanced phosphorylation at S202, S396/404, and S409 was observed compared with cells treated with vehicle or monomeric Aβ (data not shown). However, we observed a 3.2-fold increase in Alz50 immunoreactivity in neurons exposed to Aβ trimers compared with control cells (Fig. 1*A*,*B*). Applying equimolar concentrations of monomeric or dimeric Aβ did not trigger such a change. Because the epitope for Alz50 was reported to be sensitive to denaturation (Carmel et al., 1996), we compared the detection of Alz50-tau molecules in 12-month-old Htau mice (Andorfer et al., 2003) and $MAPT^{-/-}$ mice (Tucker et al., 2001). Half of the samples were denatured by boiling before loading onto PAGE gels and the other half were not subjected to heat denaturation. Using Western blotting, we did not observe differences in the ability to detect Alz50-tau in Htau mice due to sample denaturation (Fig. 1*C*,*D*). The sample denaturation step led to the detection of a prominent nonspecific ~25 kDa band by Alz50 antibodies in all specimens, including age-matched mouse $MAPT^{-/-}$ littermates (Fig. 1*C*). These results would therefore argue that heat denaturation does not prevent the accurate detection of Alz50-positive tau conformers by Western blot. Overall, these
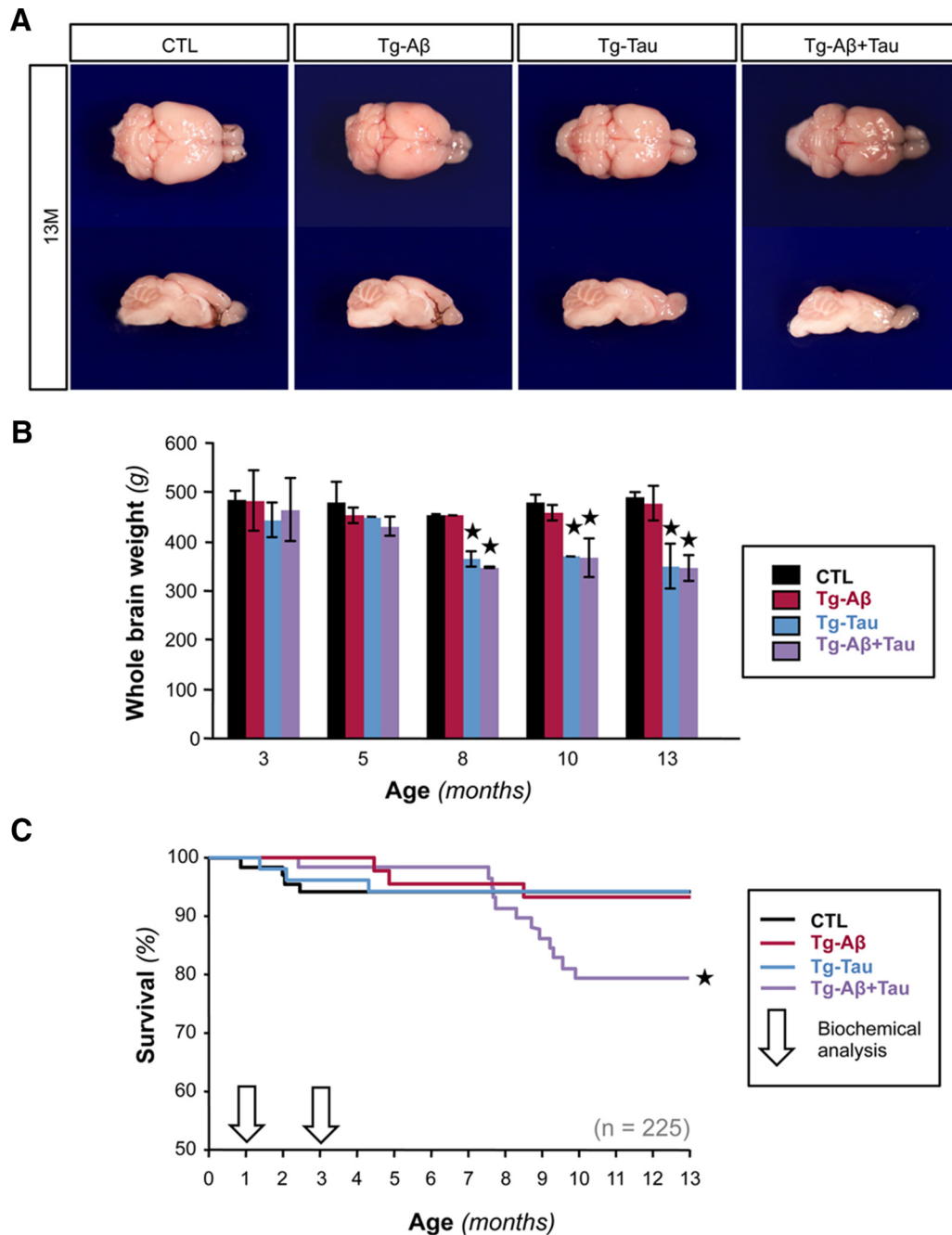
Figure 3. Tissue atrophy and survival rates in Tg-Tau + Aβ mice. **A**, Representative images show brain tissue from 13-month-old mice (top, aerial view; bottom, cross-sectional view). **B**, Mean weights of whole-brain tissue binned by age. Tg-tau and Tg-Aβ + Tau values were statistically different from both Tg-con and Tg-Aβ. Two-way ANOVA ($F_{(18, 138)} = 27.5016$, $p < 0.0001$) revealed a significant effect of age ($F_{(4, 138)} = 39.077$, $p < 0.0001$), genotype ($F_{(3, 138)} = 68.396$, $p < 0.001$), and a significant age*transgene interaction ($F_{(11, 138)} = 9.661$, $p < 0.0001$), ★$p < 0.0001$ vs control). **C**, Kaplan–Meier survival curves showing effect of the overexpression of P301L-tau on premature mortality in Tg-Aβ mice. Despite the development of substantial pathology in both Tg2576 and rTg4510 mice, neither transgenic line experienced a significant increase in mortality. In control mice ($n = 69$), we detected an approximate 5% decrease in the population within the first 400 d. This represented an expected minority of mice that died spontaneously or were killed due to colony-associated ailments or injury. This was also the case in Tg-Aβ and Tg-Tau mice despite the development of Aβ plaque and neurofibrillary tangle pathologies, respectively. In fact, we recorded little further mortality in these lines as the mice continued to age past 400 d (Tg-Aβ 91% survival at 767 d, $n = 45$, 4 deaths; Tg-Tau 94% survival at 603 d, $n = 53$, 3 deaths). In Tg-Aβ + Tau mice, a similar rate of premature death occurred up to the age of ~230 d (96% survival at 236 d, $n = 58$, 2 deaths). However, after 230 d, we observed a sudden increase in the numbers of spontaneous deaths such that survival rates decreased to 79% of expected control levels. Interestingly, increased death occurred up to the age of ~300 d, after which survival rates plateaued and further loss of Tg-Aβ + Tau mice was not recorded. Decreased survival between 230 and 300 d was a consistent phenomenon that was observed across multiple independent cohorts of aging mice and was not provoked by a change in housing conditions. Sex appeared to influence vulnerability to premature death in Tg-Aβ + Tau mice because 3 females (25%, $n = 29$) and 9 males (75%, $n = 29$) accounted for those found dead in the home cage. Because Tg-Aβ + Tau mice that lived to be >300 d of age had a moribund phenotype and were extremely inactive, all remaining +/+/+ mice assigned to the current study were killed at ~400 d. All genotyped mice in the colony were included in the analysis ($N = 251$). By log–rank comparison, only Tg-Aβ + Tau mice differed from all other groups ($\chi^2_{(3)} = 19.629$, ★$p = 0.0002$ vs control).
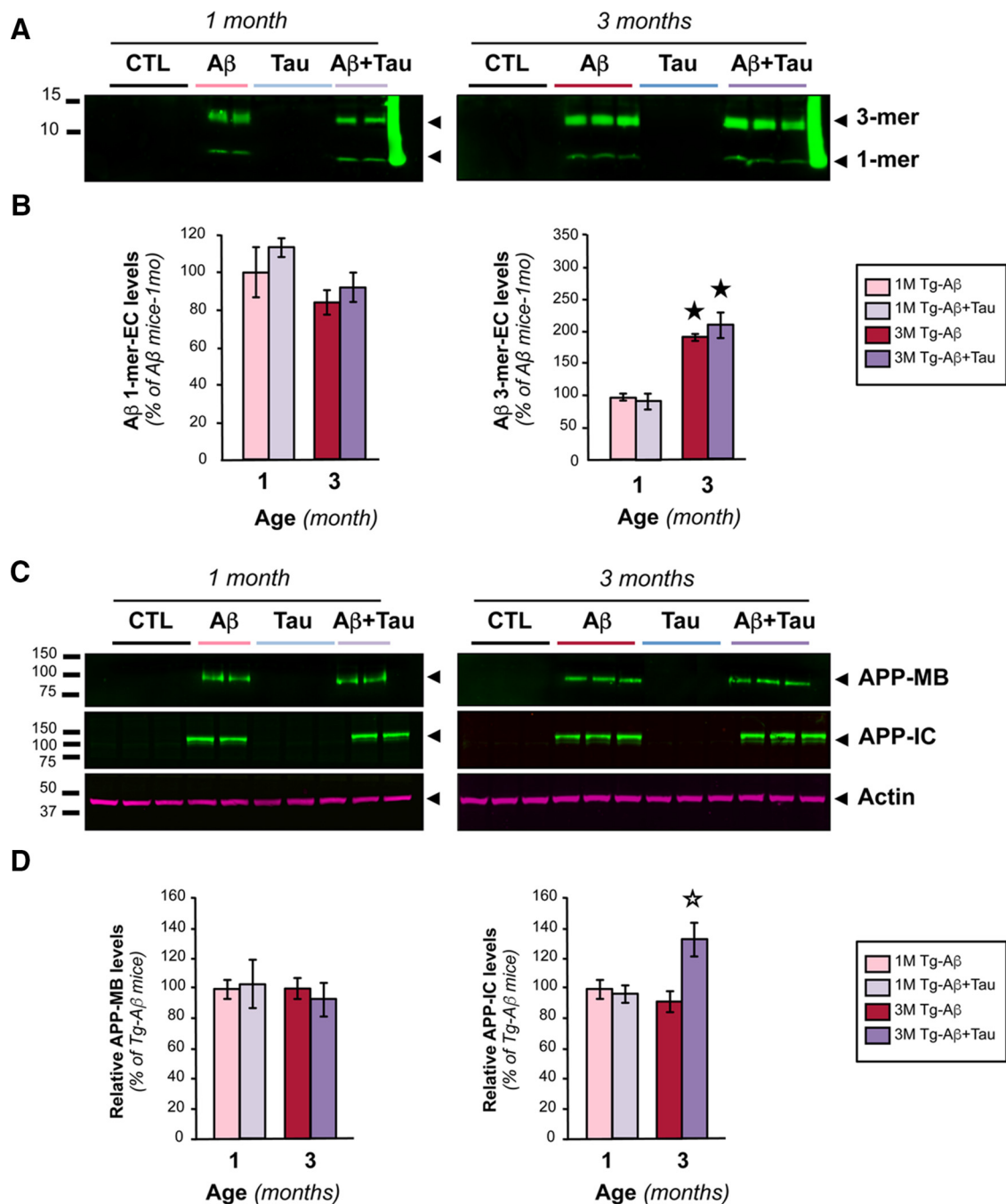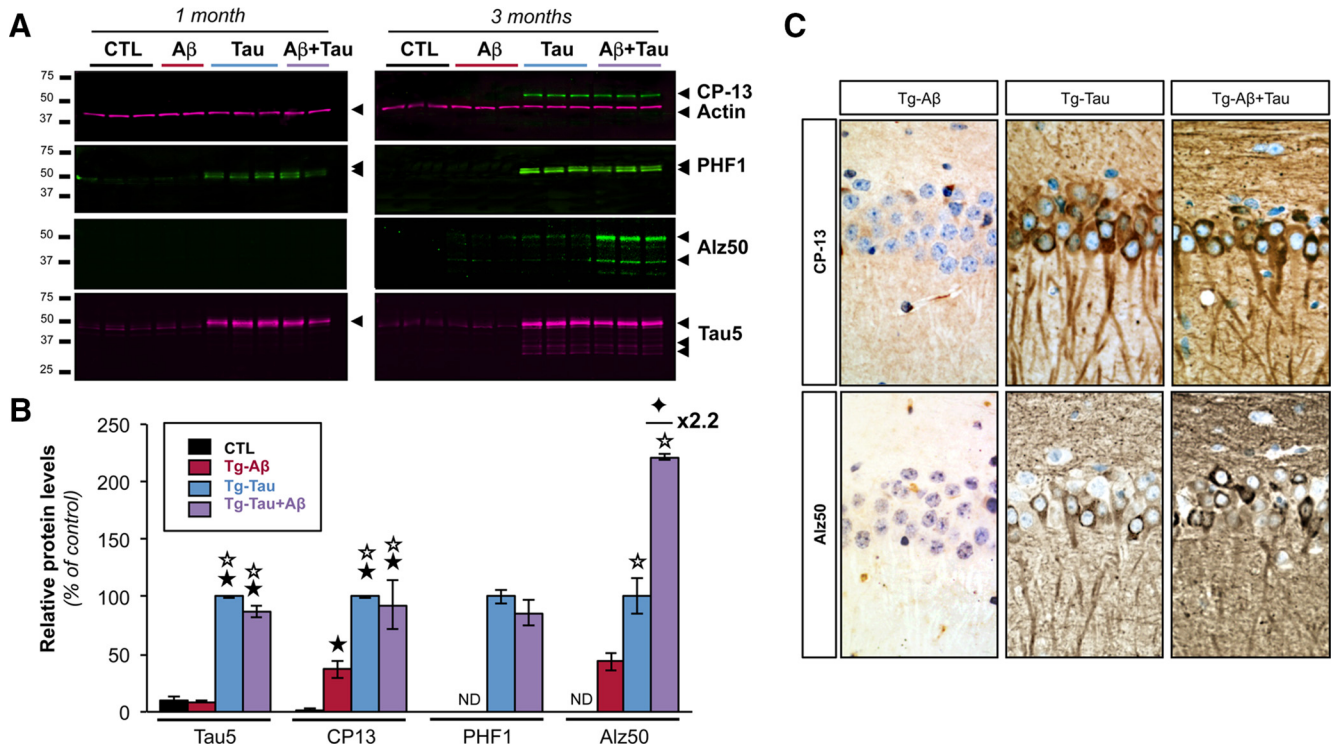
Figure 4. Age-dependent expression of soluble Aβ and APP species in forebrain tissues of bigenic Tg-Aβ+Tau mice. *A*, Representative images of soluble Aβ detected in nontransgenic (control), Tg-Aβ, Tg-Tau, and Tg-Aβ+Tau mice by immunoprecipitation with 40/42-end-specific Aβ antibodies and revealed by Western blot using 6E10. *B*, Quantitation of the levels of soluble Aβ species across genotypes revealed an ~2-fold elevation of Aβ trimers in 3-month-old mice expressing hAβ compared with younger littermates. No differences were observed between Tg-Aβ and Tg-Aβ+Tau mice. Error bars indicate the mean ± SD. Two-way ANOVA ($F_{(3, 26)} = 106.623$, $p < 0.0001$) revealed a significant effect of age ($F_{(1, 26)} = 305.939$, $p < 0.0001$), but no effect of the genotype ($F_{(1, 26)} = 1.305$, $p = 0.2651$) and no significant age*transgene interaction ($F_{(1, 26)} = 1.848$, $p = 0.1871$) (★$p < 0.05$ vs 1-month-old Aβ mice, $n = 5$–7/age/genotype). *C*, Representative images of full-length human APP detected in either membrane-enriched (top insert) or intracellular-enriched (bottom insert) fractions of nontransgenic (control), Tg-Aβ, Tg-Tau, and Tg-Aβ+Tau mice using 6E10. *D*, Densitometry analyses revealed an ~31% accumulation of intracellular APP in 3-month-old Tg-Aβ+Tau mice compared with age-matched Tg-Aβ mice. Error bars indicate the mean ± SD. ANOVA (two-way ANOVA, $F_{(3, 26)} = 13.719$, $p < 0.0001$) revealed a significant effect of age ($F_{(1, 26)} = 16.599$, $p < 0.0001$), genotype ($F_{(1, 26)} = 12.778$, $p = 0.0016$), and a significant age*transgene interaction ($F_{(1, 26)} = 7.358$, $p = 0.0124$) (★$p < 0.05$ vs 1-month-old Aβ mice, ☆$p < 0.05$ vs 1-month-old Aβ mice, $n = 5$–7/age/genotype).

findings suggested that Aβ trimers induce tau misfolding selectively.

Brain levels of Aβ trimers correlate with soluble Alz50-tau in human tissue

Previously, we reported that apparent Aβ trimers are elevated in ROS participants with MCI compared with age-matched con-

trols and subjects with AD (Lesné et al., 2013). We therefore examined whether the abundance of trimeric Aβ species was correlated with the levels of soluble Alz50-tau conformations in intracellular-enriched fractions of the inferior temporal gyrus of our MCI cohort (Fig. 2A). We observed that neither soluble monomeric nor dimeric Aβ levels were related to Alz50-tau levels (Fig. 2B,C). In contrast, we found a strong positive correlation

Figure 5. Age-dependent expression of tau species in forebrain tissues of bigenic Tg-Aβ+Tau mice. **A**, Western blot analyses of soluble tau detected in control, Tg-Aβ, Tg-Tau, and Tg-Aβ+Tau mice using CP13, PHF1, Alz50, and Tau5 antibodies displayed apparent changes in Alz50-Tau in brain tissue of bigenic mice. **B**, Quantitation of the levels of soluble tau species across genotypes revealed an ∼2.2-fold elevation of Alz-50-positive tau conformers in 3-month-old mice Tg-Aβ+Tau compared with Tg-Tau mice. Error bars indicate the mean ± SD. ANOVA ($F^{Tau5}_{(3, 28)} = 578.315$, $p < 0.0001$; $F^{CP13}_{(3, 28)} = 242.249$, $p < 0.0001$; $F^{PHF1}_{(3, 28)} = 156.129$, $p < 0.0001$; $F^{Alz50}_{(3, 28)} = 371.269$, $p < 0.0001$, respectively) followed by Student's t test (★$p < 0.05$ vs 3-month-old control mice, ☆$p < 0.05$ vs 3-month-old Tg-Aβ mice, ◆$p < 0.05$ vs 3-month-old Tg-Tau mice, $n = 5$–7/age/genotype). **C**, Although no obvious change was observed for CP13-Tau between Tg-Tau and Tg-Aβ+Tau mice by immunohistochemical analysis, changes in soluble Alz50-tau species within CA1 pyramidal neurons of Tg-Aβ+Tau mice were obvious at 3 months.

(Spearman's rho = 0.464, $p = 0.0125$, $n = 34$) between Aβ trimers measured in extracellular-enriched fractions and soluble Alz50-tau species (Fig. 2D). This observation suggested that the findings obtained *in vitro* could be pathophysiologically relevant in the context of AD.

**Elevated levels of Aβ trimers in young Tg-Aβ+Tau mice**

To attempt to create *in vivo* experimental conditions in which Aβ trimers would be elevated and that would allow us to study the relationship between Aβ and tau, we generated novel Tg-Aβ+Tau mice by crossing Tg2576 mice (Tg-Aβ) with rTg4510 mice (Tg-Tau). We chose these lines for the following reasons: (1) the brain tissue of Tg2576 mice displays relatively high levels of extracellular Aβ trimers in 1- to 3-month-old transgenic mice (Lesné et al., 2006); (2) both Tg2576 and the CKTTA activator line were in a 129S6 background strain, thereby minimizing genetic background mixing; (3) plaque deposition and tangle formation occur at ∼9 months in Tg-Aβ and at ∼4.5 months in Tg-Tau mice, respectively (Hsiao et al., 1996; Santacruz et al., 2005), allowing a fairly wide temporal window to analyze the effects of soluble forms of Aβ and tau independently of deposited amyloids; and (5) tau-induced neurodegeneration is observed at ∼5 months of age in Tg-Tau mice, providing the opportunity to study the interaction of soluble human Aβ and tau before cell loss (Ramsden et al., 2005). Once lines were generated, we first assessed whether tissue atrophy in Tg-Aβ+Tau mice was altered compared with that documented for Tg-Tau mice. Measuring whole-brain weights revealed no apparent differences between Tg-Tau and Tg-Aβ+Tau groups at any of the ages studied (3, 5, 8, 10, and 13 months; Fig. 3A,B). However, survival analyses in-

dicated that Tg-Aβ+Tau mice displayed enhanced lethality starting at ∼8 months of age (Fig. 3C). Because Tg-Aβ mice display relatively high levels of extracellular Aβ trimers in 3-month-old mice (Lesné et al., 2006), we measured the expression of Aβ and its precursor protein APP across genotypes at 1 and 3 months of age (Fig. 4). Using immunoprecipitations with 40/42-end specific antibodies of Aβ, we found that brain levels of Aβ monomers were similar at both ages in extracellular-enriched fractions of Tg-Aβ and Tg-Aβ+Tau mice (Fig. 4A,B). The abundance of trimers Aβ rose by ∼2-fold between at 1 and 3 months in APP-overexpressing mice, a 1.92- and 2.09-fold increase, respectively, in 3-month-old Tg-Aβ and Tg-Aβ+Tau brains (Fig. 4B).

Protein expression of full-length human APP pools was assessed in membrane-associated extracts and intracellular-enriched extracts by Western blotting using 6E10 (Fig. 4C). The pool of APP bound to membranes was similar between Tg-Aβ and Tg-Aβ+Tau mice (Fig. 4C,D). However, we observed a 30.58 ± 7.97% increase in APP levels in intracellular enriched lysates, whereas actin protein levels used as an internal control were unchanged (Fig. 4C,D). Similar results were obtained using aminoterminal (22C11) or carboxyterminal (APPCter-C17) antibodies against APP (data not shown), consistent with an intracellular accumulation of full-length APP.

Rise in Aβ trimers is associated with distinct conformational tau changes in vivo

Given that brain levels of apparent Aβ trimers increased by 2-fold without modifying soluble monomeric Aβ levels in 3-month-old Tg-Aβ+Tau mice, this experimental condition allowed us to test
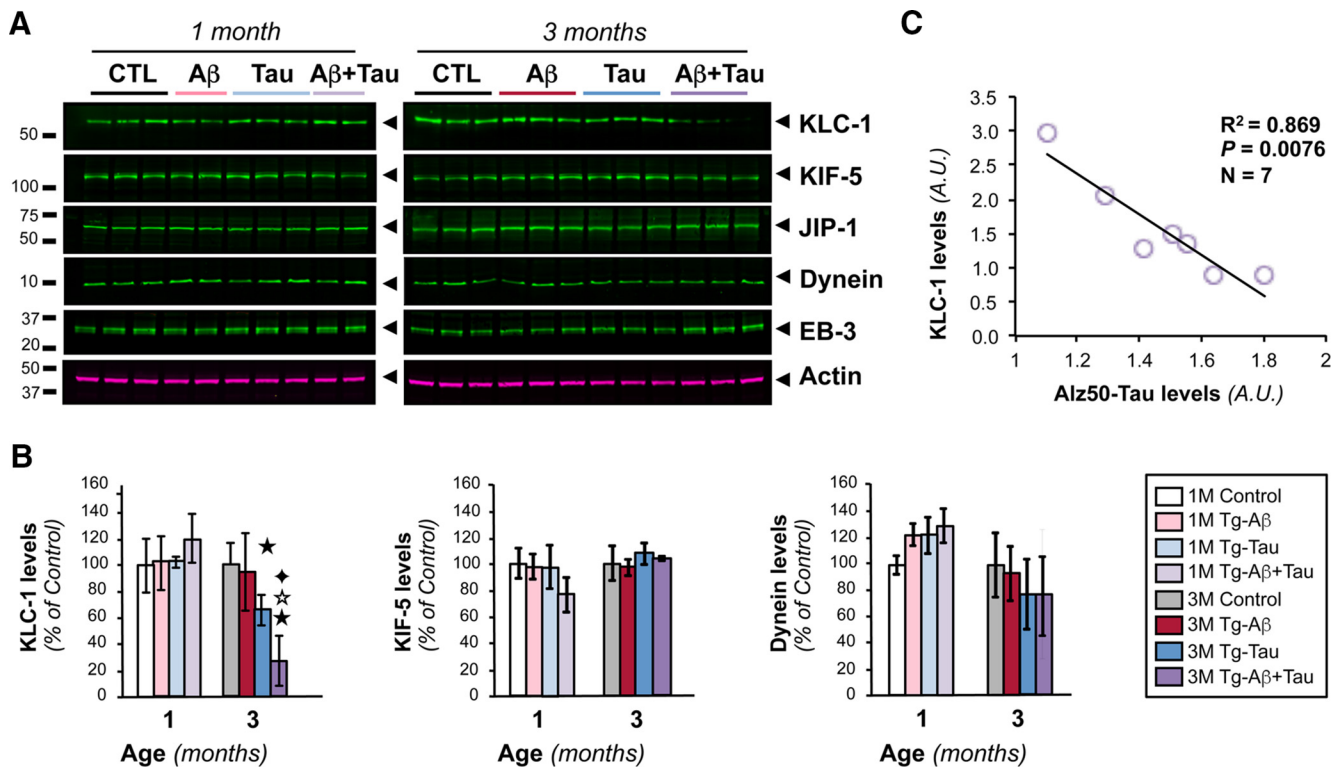
Figure 6. Selective lowering of KLC-1 in bigenic Tg-Aβ+Tau mice. *A*, *B*, Western blot analyses of proteins governing axonal transport using brain tissue of nontransgenic (control), Tg-Aβ, Tg-Tau, and Tg-Aβ+Tau mice revealed a downregulation of KLC1 in Tg-Tau mice overexpressing human tau, which was enhanced in Tg-Aβ+Tau bigenic mice (an ~2.4-fold potentiation of the effect observed) at 3 months of age. Error bars indicate the mean ± SD. Two-way ANOVA ($F^{KLC1}_{(7, 49)} = 16.972$, $p < 0.0001$) revealed a significant effect of age ($F^{KLC1}_{(1, 49)} = 24.229$, $p < 0.0001$), genotype ($F^{KLC1}_{(3, 49)} = 15.256$, $p < 0.0001$), and a significant age*transgene interaction ($F^{KLC1}_{(3, 49)} = 14.893$, $p < 0.0001$), ★$p < 0.05$ vs 3-month-old Aβ mice, ☆$p < 0.05$ vs 3-month-old Aβ mice, ◆ $p < 0.05$ vs 3-month-old Tau mice, $n = 5$–7/age/genotype). *C*, Regression analysis between KLC1 protein levels and soluble Alz50-tau conformers in 3-month-old Tg-Aβ+Tau ($F_{(1, 5)} = 24.7712$, $p = 0.0076$, $n = 7$).

whether soluble Alz50-tau molecules were exclusively augmented in this environment (Fig. 5). We first compared putative modulations in tau hyperphosphorylation and conformation observed in the early or late stages of neurodegenerative disorders involving tau. Western blotting analyses of intracellular extracts revealed no changes in CP13-Tau or PHF1-Tau levels between Tg-Tau and Tg-Aβ+Tau mice (Fig. 5*A*,*B*), indicating that Aβ was not potentiating these disease-related tau changes. However, we detected a 2.2-fold elevation of soluble tau conformers detected with Alz50 in bigenic Tg-Aβ+Tau mice compared with Tg-Tau littermates (Fig. 5*A*,*B*). Importantly, total tau levels measured with Tau5 antibodies remained unchanged across Tg-Tau and Tg-Aβ+Tau mice, suggesting that the conformation of tau was altered in the presence of constant expression. To support these biochemical analyses of tau, we performed immunohistochemical studies on brain sections from Tg-Aβ, Tg-Tau and Tg-Aβ+Tau mice using CP13 and Alz50 antibodies (Fig. 5*C*). Although CP13-Tau immunoreactivity within the CA1 hippocampal neurons appeared comparable between Tg-Tau and Tg-Aβ+Tau mice (Fig. 5*C*, top row), a clear increase in Alz50 staining was readily observed in the neuronal soma of CA1 pyramidal neurons of bigenic mice compared with Tg-Tau mice (Fig. 5*C*, bottom row). Overall, the 2-fold elevation of Aβ trimers in 3-month-old Tg-Aβ+Tau mice is associated with a selective ~2-fold increase in tau misfolding.

Twofold elevation in Aβ trimers/Alz50-tau is associated with selective deficits in KLC1 governing anterograde axonal transport
Because tau is critical for axonal transport in the presence of synthetic Aβ oligomers *in vitro* (Vossel et al., 2010) and APP

appeared to accumulate intracellularly in 3-month-old Tg-Aβ+Tau mice, we hypothesized that the accumulation of soluble Alz50-tau molecules associated with the elevation of Aβ trimers in bigenic mice could alter the proteins governing anterograde axonal transport. We therefore assessed the expression levels of motor proteins responsible for anterograde axonal transport, KLC-1, KIF-5, and the scaffold protein JIP-1, and for retrograde axonal transport, dynein. At 1 month of age, the abundance of the measured proteins appeared unaffected (Fig. 6*A*,*B*). Two months later, the expression of KLC-1 was reduced to 65.82 ± 11.51% in forebrain tissue of Tg-Tau mice compared with nontransgenic mice. Importantly, KLC-1 expression further dropped to ~28% in bigenic Tg-Aβ+Tau mice, a 2.4-fold decrease compared with Tg-Tau mice (Bonferroni-corrected *t* test after ANOVA, $p = 0.0012$, $n = 5$–6/age/genotype). Not all proteins regulating anterograde axonal transport were impaired because neither KIF-5 nor JIP-1 protein expression changed across genotypes. In addition, the brain levels of the motor protein dynein did not seem to be modified at the ages and genotype tested (ANOVA, $p > 0.05$, $n = 5$–6/age/genotype). We next assessed whether KLC-1 expression levels were related to those of Alz50-tau species in bigenic Tg-Aβ+Tau mice and found a negative correlation between these two variables (Fig. 6*C*). These data suggest the possibility that the increases in Alz50-tau and Aβ trimers observed in bigenic Tg-Aβ+Tau mice might trigger this change in KLC-1 expression.

To determine whether a similar association was observed in human brains, we measured KLC-1 protein expression in the ROS cohort (Fig. 7*A*). The relative expression of KLC-1 was reduced in the AD group, consistent with earlier reports (Mo-

A

B

C
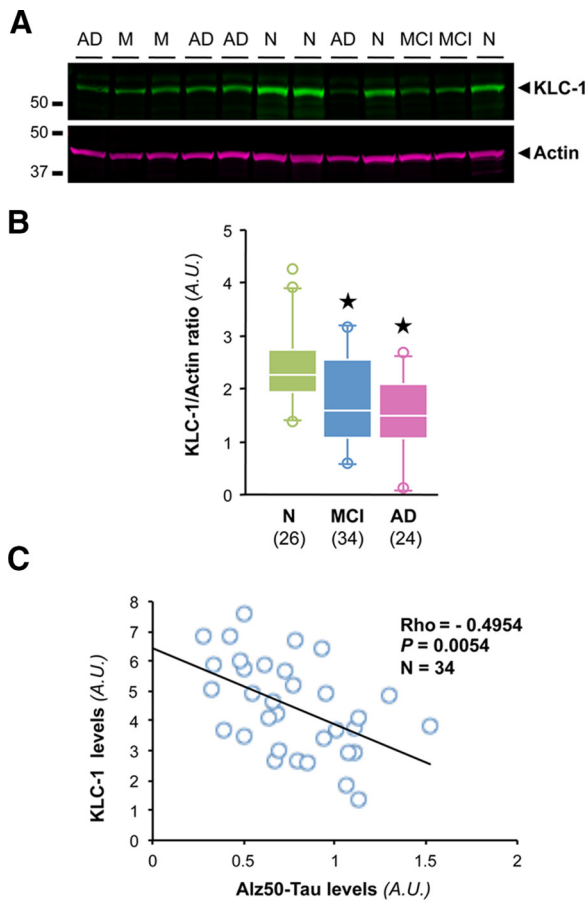
Figure 7. Relationship between KLC-1 and Alz50-tau species in MCI brains. **A**, Western blot analysis of KLC-1 protein levels in the ROS cohort analyzed. Actin was used as an internal standard. The images shown only correspond to a subset of the specimens studied (12 of 84). **B**, Box plot of the relative levels of KLC-1 across clinical groups. Italicized numbers in parentheses indicate group sizes. No cognitive impairment ("N") is shown in green, MCI is shown in blue, and AD in indicated by pink boxes. The bar inside the box indicates the median; the upper and lower limits of boxes represent the 75th and 25th percentiles, respectively; and bars flanking the box represent the 95th and fifth percentiles (★$p < 0.05$ vs N, Kruskal–Wallis test ($\chi^2_{(2)} = 13.499$, $p = 0.0012$), followed by Mann–Whitney U test, N = 84. **C**, Regression analyses between KLC1 protein levels and soluble Alz50-tau conformers in ROS MCI measured (Spearman's rho correlation, $n = 34$).

rel et al., 2012), and in the MCI group (Fig. 7B). Because trimeric Aβ levels are highest in MCI subjects from the ROS cohort (Lesné et al., 2013), we examined the relationship between KLC-1 and Alz50-tau in this group. In this context, the abundance in KLC-1 protein was inversely correlated with tau conformers detected by Alz50 (Fig. 7C).

Aβ trimers trigger decreases in KLC-1 protein mediated by tau conformers

To evaluate directly the potential of Aβ trimers to lower KLC-1 protein levels, we treated mouse cortical neurons with either brain-derived Aβ trimers (1–2 nM for 60 min) or vehicle and examined KLC-1 and KIF-5 expression in these cultured primary cells (Fig. 8A,B). Upon treatment with Aβ trimers, KLC-1 expression was remarkably reduced to 47.42 ± 5.64% compared with control neurons ($p < 0.001$, $n = 6–8$/treatment). Consistent with the in vivo findings reported above, no changes in KIF-5 protein expression were observed. Because PrP^C and tau have been proposed to mediate oAβ-induced toxicity (Roberson et al., 2007; Laurén et al., 2009; Vossel et al., 2010; Larson et al., 2012;

Um et al., 2012), we next assessed whether PrP^C (Fig. 8C,D) or tau gene products (Fig. 8E,F) were mediating the lowering of KLC1 induced by Aβ trimer exposure in Prnp-null or Mapt-null cortical neurons. In agreement with our previous study indicating that Aβ dimers, but not Aβ trimers, were coimmunoprecipitating with PrP^C (Larson et al., 2012), deletion of the gene encoding for PrP^C did not rescue the decrease in KLC-1 proteins levels when Aβ trimers were applied to cells (Fig. 8C,D). Similarly to WT neurons, KIF-5 expression was unaltered in Prnp-null neurons (Fig. 8D).

To determine whether changes in tau induced by AD brain-purified Aβ trimers preceded KLC-1 reductions, we applied Aβ trimers or vehicle onto Mapt-null cortical neurons (Fig. 8E,F). Contrary to the 50–55% reduction in KLC-1 observed in WT or Prnp-null neurons, KLC-1 protein levels were unaffected by Aβ trimers in tau-deficient neurons. This result demonstrated that Aβ-induced deficits in KLC-1 required expression of tau. Finally, to establish that soluble Alz50-tau conformers were necessary to mediate the selective decrease in KLC-1 triggered by trimeric Aβ application, we preconditioned neurons by delivering Alz50 or control IgM antibodies intracellularly using Chariot technology 30 min before exposure to Aβ trimers (Fig. 8G–I). Intraneuronal delivery of control immunoglobulins did not alter KLC-1 expression (Fig. 8G,H), arguing against a possible nonspecific effect of antibody delivery. When the Chariot shuttling reagent was applied alone, application of Aβ trimers led to an ∼44% lowering in KLC-1, similar to what was observed in naive WT neurons (Fig. 8A,B). In contrast, Alz50-pretreated neurons appeared to be protected from the effect of Aβ trimers on KLC-1 (Fig. 7H). These findings established directly that Alz50-positive conformers were required to alter KLC-1 protein levels in the presence of Aβ trimers.

Discussion

Despite accumulating evidence supporting the concept that oligomeric forms of Aβ constitute the biological vessel responsible for synaptic dysfunction in AD, the exact role of each assembly of Aβ remains unknown and shrouded in controversy (Benilova et al., 2012; Lesné, 2013). Our group recently suggested that Aβ molecules identified as AD brain-tissue-purified Aβ dimers and trimers by immunological, electrophoretic, and liquid-phase separation techniques activate the kinase Fyn similarly in vitro (Larson et al., 2012). In the same report, we proposed that the cellular form of the prion protein PrP^C acted as a transducing receptor for Aβ dimers, but not Aβ trimers, thereby inducing the hyperphosphorylation of tau at Y18 (Larson et al., 2012). Importantly, neither soluble Aβ*56 nor protofibrillar Aβs nor Aβ monomers triggered the phosphorylation of Fyn/tau under similar conditions, suggesting that not all Aβ oligomers induce the same intracellular signaling pathways. This notion is particularly important in the context of earlier reports demonstrating that the respective abundance of apparent Aβ dimers, trimers, and Aβ*56 varies across clinical groups in humans (Shankar et al., 2008; Lesné et al., 2013). One likely consequence of these studies is that each oligomeric Aβ assembly might stimulate distinct cellular pathways during the course of the disease, all of which (maybe sequentially or synergistically) constitute the key molecular events underlying AD.

The hypothesis developed above led us to assess whether additional selective pathological changes of tau might occur in primary cortical neurons exposed to oAβ species purified from biologically relevant brain tissue. Surprisingly, we found that soluble Alz50-positive tau conformers were specifically induced
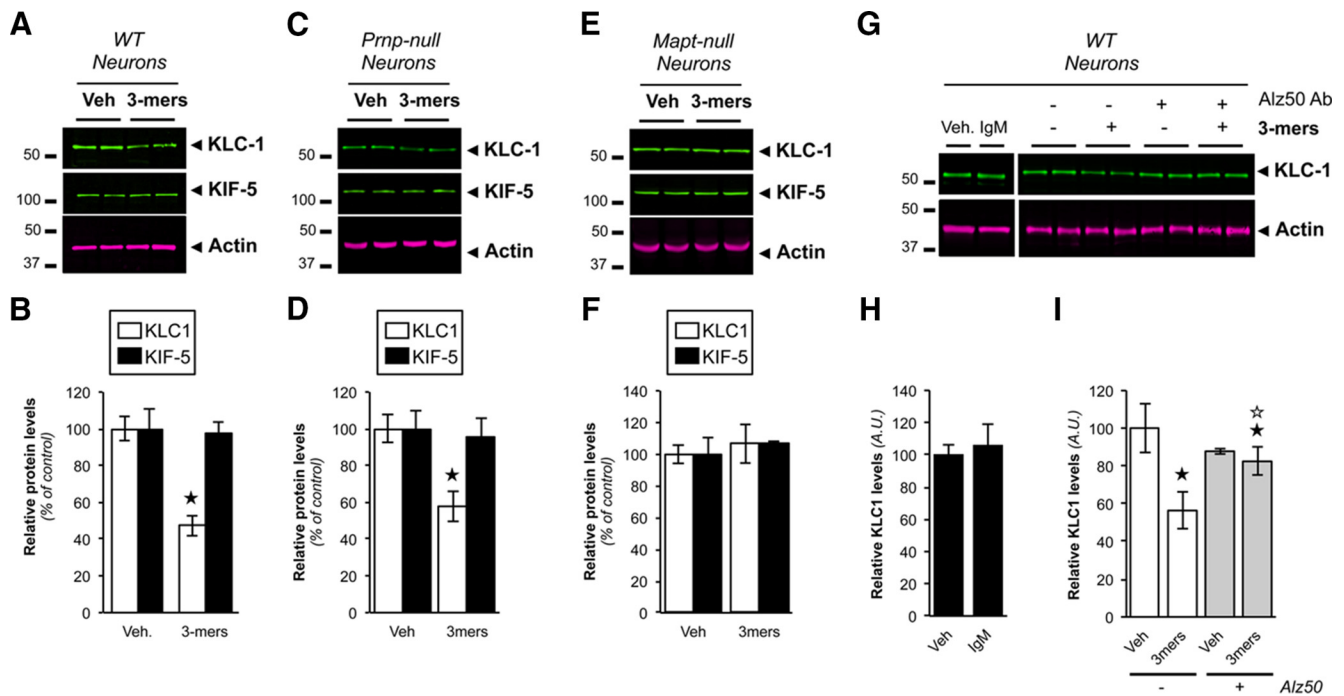
Figure 8. Trimeric Aβ-induced selective lowering of KLC-1 is dependent on tau conformers in primary cortical neurons. Mouse primary cortical neurons were exposed to either vehicle or 1 nM trimeric Aβ for 1 h. **A**, **B**, Protein levels of KLC1 were altered in cultured neurons after exposure with Aβ trimers, whereas KIF-5 and actin were unchanged. Error bars indicate the mean ± SD (*t* test, $t_{(7.88)} = 9.476$, $p < 0.0001$, ★$p < 0.05$ vs vehicle, $n = 6 – 8$/treatment). **C**, **D**, In *Prnp*-null primary neurons, similar findings were observed to those described in **A** and **B**. Error bars indicate the mean ± SD (*t* test ($t_{(8)} = 9.147$, $p < 0.001$), ★$p < 0.05$ vs vehicle, $n = 8$/treatment). **E**, **F**, In *Mapt*-null neurons, KLC1 proteins levels were not downregulated when Aβ trimers were applied to cells. Error bars indicate the mean ± SD (*t* test, ★$p < 0.05$ vs vehicle, $n = 6 – 8$/treatment). **G–I**, Effect of pretreatments with IgM (**H**) or Alz50 (**I**) delivered using Chariot delivery technology on KLC1 deficits triggered by Aβ trimers. Error bars indicate the mean ± SD (*t* test, $t_{(14)} = 0.548$, $p > 0.05$, ★$p < 0.05$ vs vehicle, $n = 6 – 8$/treatment; ANOVA, $F_{(3, 25)} = 63.227$, $p < 0.0001$, followed by Student's *t* test, ★$p < 0.05$ vs vehicle, ☆$p < 0.05$ vs Aβ 3-mers, $n = 6 – 8$/treatment for **H** and **I**, respectively).

upon treatment with Aβ trimers. The Alz50 antibody is a monoclonal antibody that was initially found to stain fibrillar tau pathology in AD brain tissue (Wolozin et al., 1986; Wolozin and Davies, 1987). Its epitope was subsequently identified to recognize a folded conformation of tau containing amino acids 2–10 and 312–342 (Carmel et al., 1996; Kopeikina et al., 2013). This folding change was not detected in cells exposed to other soluble forms of Aβ tested here (i.e., Aβ monomers, dimers, Aβ*56, and Aβ protofibrils). This finding led us to question whether similar changes could be observed *in vivo* when Aβ trimers are abundant. Based on our prior characterization of the Tg2576 mouse line (Lesné et al., 2006; Lesné et al., 2008), we anticipated that crossing Tg2576 with rTg4510 mice would allow us to studying a potential interaction between Aβ trimers and tau pathological changes. In very young bigenic mice at 3 months of age, in which forebrain levels of apparent trimeric Aβ were doubled, we indeed observed a selective change in tau detected by Alz50. Perhaps not coincidentally, it is interesting to us that the amplitude of the change in the elevation of Aβ timers between 1- and 3-month-old mice (a 2.09-fold increase) is similar to the amplitude in the change of soluble tau conformers detected by Alz50 (a 2.2-fold increase). In parallel, full-length APP seemed to accumulate in the intracellular-enriched protein fractions of Tg-Aβ+Tau mice, suggesting that APP trafficking might be compromised. Because synthetic preparations of Aβ oligomers can alter tau-mediated axonal transport *in vitro* (Vossel et al., 2010), we tested whether increases in these soluble forms of Aβ and tau were associated with modulations in the expression of proteins regulating axonal transport. Consistent with previous observations indicating that alterations of fast axonal transport represent an early pathological event (Stokin et al., 2005;

Muresan and Muresan, 2009), KLC-1 protein levels, which were already impaired in Tg-Tau brains, were further reduced in Tg-Aβ+Tau, whereas KIF-5, JIP-1, and Dynein protein levels appeared to be unaltered. This observation suggested that Aβ trimers/Alz50-tau might cooperate in selectively altering KLC-1-dependent mechanisms. To demonstrate that Aβ trimers were the initiator molecule in that cascade, we treated cultured neurons with Aβ trimers purified from AD brain tissue and validated that neuronal exposure with this oligomeric assembly was sufficient to reduce KLC-1 expression selectively. Although other groups have reported that uncharacterized synthetic mixtures of Aβ oligomers alter tau-mediated axonal defects (Vossel et al., 2010), we believe that our *in vitro* and *in vivo* findings are the first to demonstrate that a specific endogenous oligomeric Aβ assembly alters proteins governing axonal transport. To our knowledge, this report also constitutes the first description of selective conformational changes in tau induced by different Aβ oligomers. Finally, taking into consideration that brain levels of Aβ trimers appear to be highest in subjects with MCI and lower in AD (Lesné et al., 2013), these results highlight the importance of considering the impact of each Aβ oligomer individually. Indeed, we predict that a strategy interfering with Aβ trimers/Alz50-tau would only prevent axonal transport alterations during the prodromal stage of AD. Based on this current work and earlier reports suggesting that different oAβs trigger distinct pathological changes during disease progression (Larson et al., 2012; Lesné et al., 2013), we believe that separate therapies aimed at disrupting specific oAβ/tau interactions will need to be considered to treat patients at the preclinical AD, MCI, or AD stages.

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