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Tau pathogenesis is promoted by A β 1-42 but not A β 1-40

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Abstract

Background: The relationship between the pathogenic amyloid β -peptide species A β 1-42 and tau pathology has been well studied and suggests that A β 1-42 can accelerate tau pathology in vitro and in vivo. The manners in which A β 1-40 interacts with tau remains poorly understood. In order to answer this question, we used cell-based system, transgenic fly and transgenic mice as models to study the interaction between A β 1-42 and A β 1-40.

Results: In our established cellular model, live cell imaging (using confocal microscopy) combined with biochemical data showed that exposure to A β 1-42 induced cleavage, phosphorylation and aggregation of wild-type/full length tau while exposure to A β 1-40 didn't. Functional studies with A β 1-40 were carried out in tau-GFP transgenic flies and showed that A β 1-42, as previously reported, disrupted cytoskeletal structure while A β 1-40 had no effect at same dose. To further explore how A β 1-40 affects tau pathology in vivo, P301S mice (tau transgenic mice) were injected intracerebrally with either A β 1-42 or A β 1-40. We found that treatment with A β 1-42 induced tau phosphorylation, cleavage and aggregation of tau in P301S mice. By contrast, A β 1-40 injection didn't alter total tau, phospho-tau (recognized by PHF-1) or cleavage of tau, but interestingly, phosphorylation at Ser²⁶² was shown to be significantly decreased after direct inject of A β 1-40 into the entorhinal cortex of P301S mice.

Conclusions: These results demonstrate that A β 1-40 plays different role in tau pathogenesis compared to A β 1-42. A β 1-40 may have a protective role in tau pathogenesis by reducing phosphorylation at Ser²⁶², which has been shown to be neurotoxic.

Keywords: A β 1-42, A β 1-40, tau, Alzheimer's disease, Aggregation, Phosphorylation, Cleavage

Background

Two major hallmarks of Alzheimer's disease (AD) are the accumulation of the amyloid- β peptides (A β) into extracellular plaques and the formation of intracellular neurofibrillary tangles (NFTs) composed mainly of the protein tau. A β exists as two main species, A β 1-42 and A β 1-40, and the unique relationship between each of these peptides on tau pathology has not been adequately addressed. It is generally believed that A β accrual in brain is an early event in the pathogenesis of AD, preceding significant tau pathology. Intracerebral administration of synthetic A β 1-42 fibrils into P301L mice (mutant human tau transgenic mice) induced tau hyperphosphorylation and local neurofibrillary tangles [1,2]. Natural

A β oligomers, specifically dimers, isolated from a human AD brain, were sufficient to induce tau hyperphosphorylation at AD-relevant epitopes as well as microtubule disruption and neuritic degeneration [3]. These experiments were done either with A β 1-42 or a mixture of A β 1-42 and A β 1-40; however, increasing evidence suggests that A β 1-40 and A β 1-42 differentially contribute to the disease process [4]. BRI-A β 40 mice, a model that exclusively expresses high levels of A β 1-40, do not develop amyloid pathology in the form of diffuse or neuritic A β plaque. BRI-A β 42 mice, on the other hand, a model that exclusively expresses A β 1-42, and at levels 10-fold lower than BRI-A β 40 mice express A β 1-40, developed amyloid deposits in the cerebellum as early as 3 months of age [4]. By crossing BRI-A β 40 or BRI-A β 42 mice with Tg2576 (APP^{swe}, K670N + M671L) mice, it has been shown that A β 1-42 and A β 1-40 have opposing effects on amyloid deposition; A β 42 promotes amyloid

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deposition, while A β 40 inhibits it [5]. In addition, inhibition of angiotensin-converting enzyme (ACE), which converts A β 42 to A β 40, enhanced brain amyloid deposition in Tg2576 mice [6]. Emerging evidence therefore indicates a protective role for A β 1-40 in AD pathogenesis. One such theory suggests that A β 1-40 may act to stabilize A β 1-42 monomers by competing for binding sites on pre-existing A β 1-42 aggregates [7] and thereby inhibiting further aggregation of A β 1-42 [8].

A β 1-42 has also been shown to increase the phosphorylation and aggregation of tau both in vivo [1] and in vitro [3,9]; however, the interaction between A β 1-40 and tau has not, to our knowledge, been explored. Increasing experimental evidence on differential (in some cases opposite) roles that A β 1-40 and A β 1-42 may play in amyloid deposition raises the question of whether A β 1-40 alters tau phosphorylation and/or aggregation in the same manner as A β 1-42. Here, we address this issue by using: 1) a cell-based model to study the effects of A β 1-42 and A β 1-40 on tau aggregation using live cell imaging; 2) a transgenic *Drosophila* model to examine the direct impact on cytoskeleton structure caused by A β 1-42 and A β 1-40; 3) a tau transgenic mouse model to compare the effects of A β 1-42 and A β 1-40 on tau pathogenesis. Here we show that A β 1-42 induces tau phosphorylation, aggregation and cleavage whereas A β 1-40 does not.

Results

Full-length tau aggregates quickly in SHSY5Y and C17.2 cells, but not in N2a cells

To explore the capability of wild-type full-length tau (2N4R, Tau441) to aggregate in living cells, SHSY5Y, C17.2 and N2a cells were plated on an ibidi u-Slide 8 well chamber and transiently transfected with Tau441-YFP (2N4R—longest form of human tau) using lipofectamine 2000. Twenty four hours later, live cell images were taken using a confocal microscope (see Methods). Darkfield and phase contrast views of cells were shown side to side. Darkfield images showed better fluorescence signal (especially for intracellular aggregates) and phase contrast provided images for the whole cell. As shown in Figure 1A, after 24 h we observed the aggregation of full length tau in the vesicles of C17.2 cells and SHSY5Y cells (Red arrow in Figure 1B) but not in N2a cells, suggesting cellular specificity for the aggregation of tau. This is the first study to show that wild-type full-length tau can form aggregates without the addition of exogenous recombinant tau fibrils. By quantifying the fluorescent intensity of YFP linked to Tau441, we calculated the transfection efficiency in N2a cells to be approximately 85% compared to 35% for C17.2 and 5% for SHSY5Y cells.

To examine the aggregation of Tau441 in N2a cells over time, live cell images were taken at 24 h, 48 h and

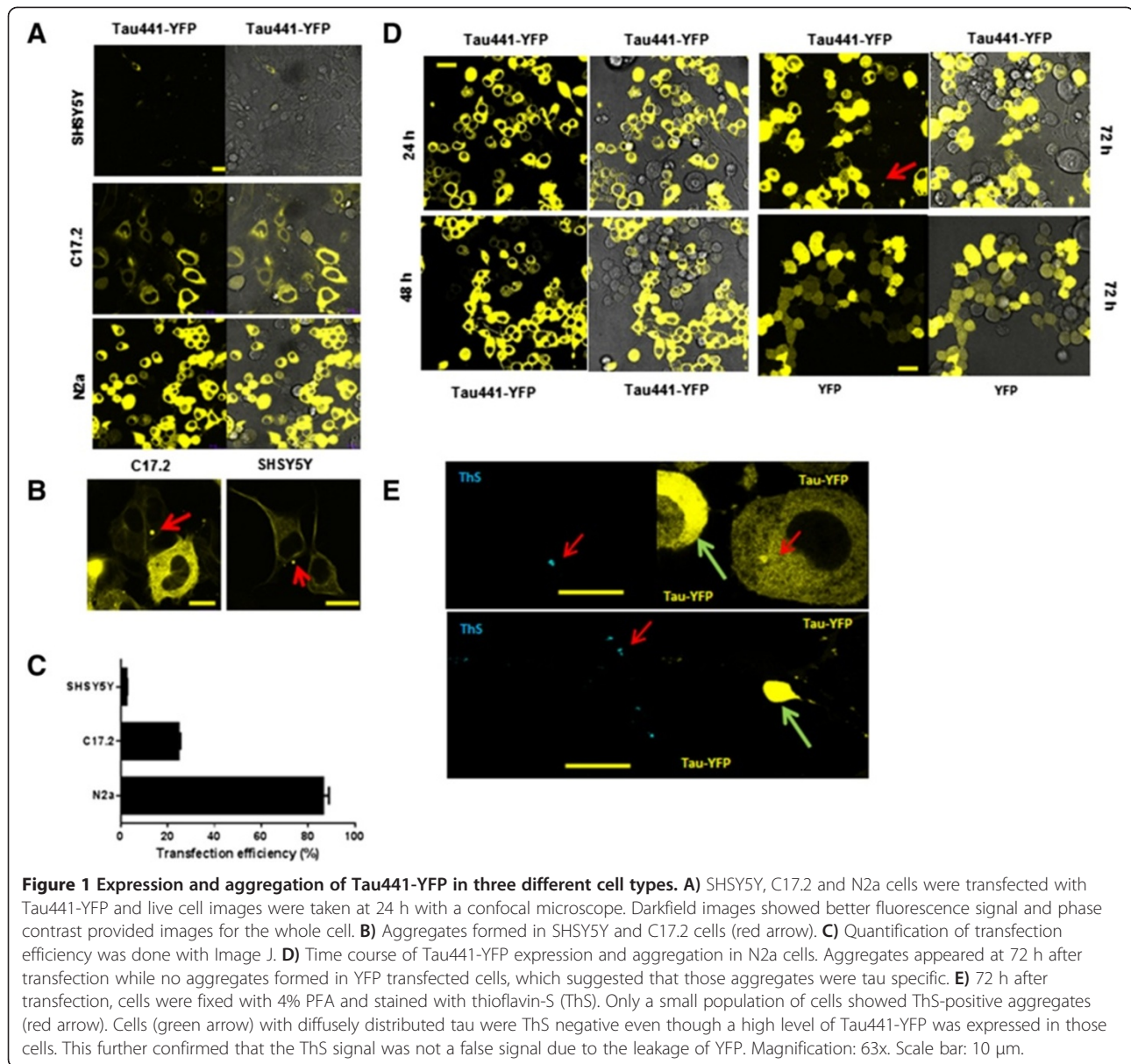
72 h after transfection. Tau aggregates were seen in N2a cells 72 h after transfection (Figure 1D), but not in YFP expressing cells, indicating that the aggregates seen in tau-YFP transfected N2a cells were not YFP-dependent, but specific for tau. Thioflavin-S (ThS) was used to determine if tau aggregates were in β -pleated sheets structure as seen in tau transgenic mice and human AD brain sections. Seventy two hours after transfection with Tau441-YFP, N2a cells were fixed and stained with ThS. As shown in Figure 1E, ThS staining completely overlapped with the large tau aggregates highlighted with YFP, but not with diffusible tau in cytoplasm.

A β 1-42 increases the level of insoluble tau while A β 1-40 has no effect

In vivo, A β 1-42 is more prone to forming amyloid plaque than A β 1-40. In fact, A β 1-40 has been shown to have a protective effect by reducing the fibrilization of A β 1-42 [5]. Using the cell-based model we established above, we first determined the respective influence of A β 1-40 and A β 1-42 on tau insolubility. N2a cells were transfected with Tau441-YFP in the presence of 200 nM soluble A β 1-40 or A β 1-42. Twenty four hours after transfection, live cell images were taken using confocal microscopy and there was no difference in the total fluorescence intensity between the groups (Figure 2A). To assess the effects of A β on tau insolubility, cells were fixed with 4% PFA containing 1% Triton-X100 for 15 min to remove soluble tau from the cells. Images shown in Figure 2B, where the fluorescent signal represents insoluble tau, indicate that there was much more insoluble full-length tau in the presence of A β 1-42 when compared to A β 1-40 and non-treated cells. Quantification of the fluorescence intensity in these images was done using Image J (Figure 2C). Cytotoxicity was found in A β 1-42 treated N2a cells at 72 hr but not in A β 1-40 treated cells (data not shown).

A β 1-42 induces cleavage, phosphorylation and aggregation of tau while A β 1-40 does not

To understand the underlying mechanisms that contribute to the unique roles of A β 1-42 and A β 1-40 on tau pathology, N2a stable cell line that overexpresses Tau441-YFP was established. Twenty four hours after treatment with either A β 1-42 or A β 1-40, the cells were homogenized on ice in 1% Triton-PBS buffer, followed by 1% SDS-PBS buffer. Western blots analysis of Triton and SDS extract using anti-tau antibodies showed that A β 1-42 treatment resulted in increased phosphorylation at Ser²⁶², as recognized by the pS262 antibody, in both Triton and SDS soluble fractions as compared with N2a cells that did not receive A β treatment (Figure 3A and C). A slight decrease in the pS262 signal was observed in the SDS fraction from A β 1-40 treated cells. Phosphorylation at Ser²⁶² strongly reduces



binding of tau to microtubules and has been shown to be a key phosphorylation site implicated in the loss of function of tau in cytoskeleton stabilization [10]. Ser³⁹⁶/Ser⁴⁰⁴ is important phosphorylation site that is recognized by the PHF-1 antibody. In A β 1-42 treated cells we also observed a 3-fold increase in total tau and phosphorylation at the PHF-1 epitope (Ser³⁹⁶/Ser⁴⁰⁴) in the SDS fraction, but we observed no change in A β 1-40 treated cells (Figure 3B and D). We also observed an increase in Tau421 (cleavage of tau at Asp⁴²¹) in the Triton fraction after A β 1-42 treatment as visualized with the tau-C3 antibody (Figure 3A and B). No tau-C3 signal was detected in the SDS fraction. Our data are consistent with previous studies on the accelerating effect of A β 1-42 on tau pathology. To our knowledge,

this is the first study to show that A β 1-40 has no effect on tau phosphorylation, cleavage and aggregation.

Activation of GSK3 β and caspase-3 by A β 1-42 but not by A β 1-40

GSK3 β phosphorylates tau at many sites including Ser²⁶², Ser³⁹⁶ and Ser⁴⁰⁴. To understand the underlying mechanism responsible for the differences in tau phosphorylation induced by A β 1-42 and A β 1-40 treatments, total GSK3 β and phosphorylated-GSK3 β (p-GSK3 β) levels were examined by western blot. Phosphorylation of GSK3 β at Tyr²¹⁶ increases the activity of GSK3 β , which leads to more tau phosphorylation. As shown in Figure 3E, A β 1-42 treatment increased p-GSK3 β (Y216) without changing the

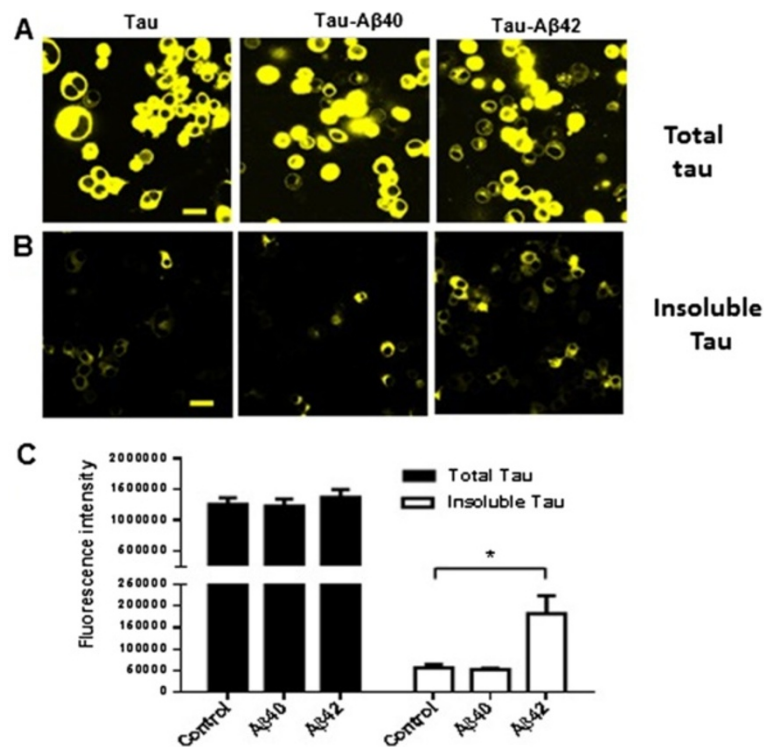


Figure 2 Aβ1-42 induces intracellular full length tau (Tau441-YFP) aggregation in N2a cells. **A**) N2a cells were transfected with Tau441-YFP and treated with 200nM Aβ1-40 or Aβ1-42. 24 h later, cells were imaged directly under a confocal microscope. There was no difference in the expression of tau between the groups. **B**) Cells were extracted with 1% Triton during fixation, which left only insoluble tau in the cells. As shown in Figure B, Aβ1-42 treatment increased the level of insoluble full-length tau, but Aβ1-40 did not. **C**) Quantification of fluorescence intensity was done with Image J. Magnification: 63x, scale bar: 10 μm. Three individual experiments were done for each condition. *p < 0.05.

total GSK3β level. By contrast, Aβ1-40 treatment resulted in no change in either GSK3β or p-GSK3β.

Aβ1-42 treatment has also been reported to activate pro-caspase-3 [11] and enhance the cleavage of tau at Asp⁴²¹, which results in accelerated aggregation. Consistent with these results, we also found increased Tau421 in Aβ1-42 treated cells. Western blots with an anti-active caspase-3 antibody revealed higher levels of active caspase-3 following Aβ1-42 treatment but not following Aβ1-40 treatment (Figure 3E and F).

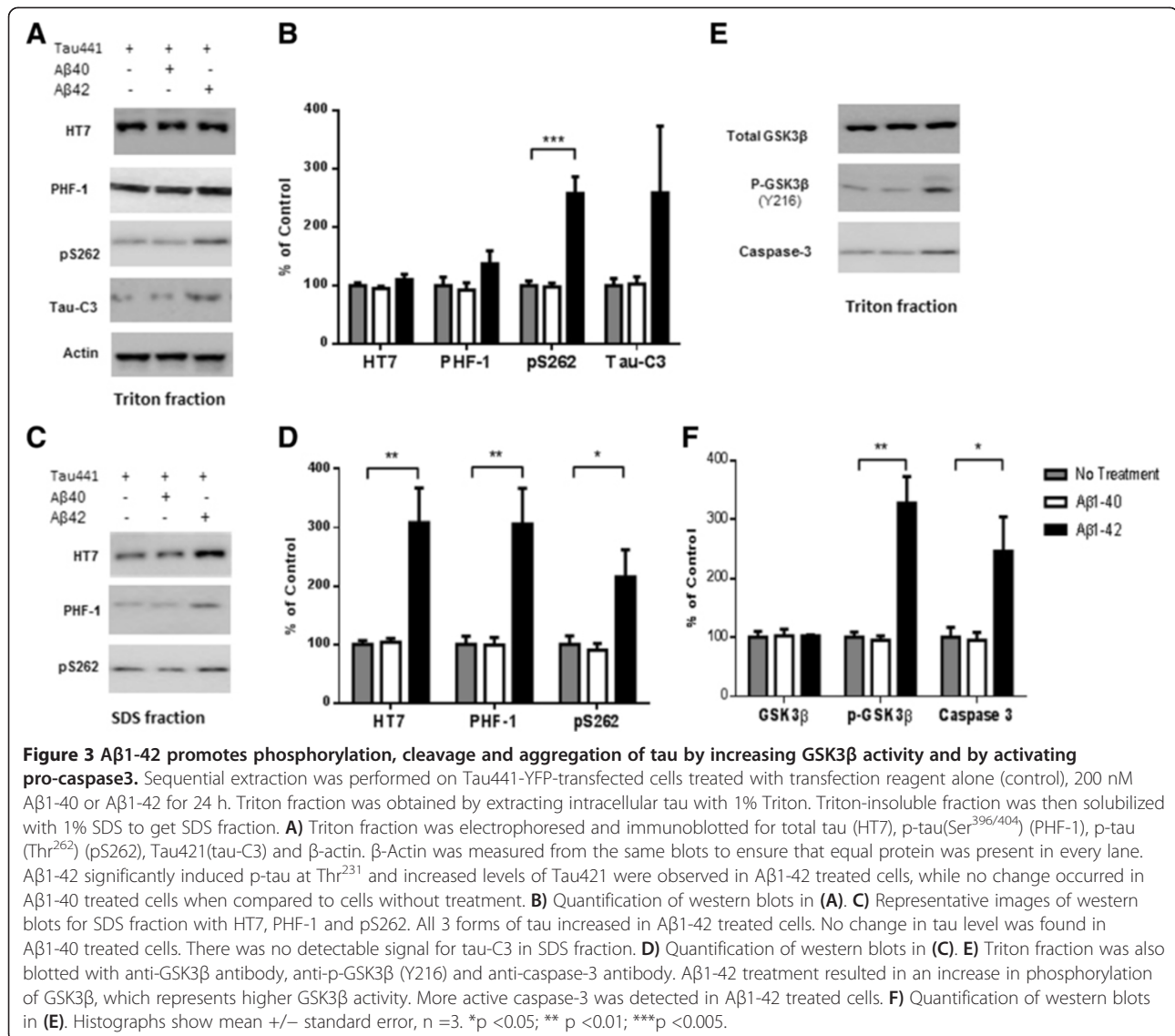
Aβ1-42, but not Aβ1-40, interferes with cytoskeleton formation in tau transgenic flies

Tau plays an important role in microtubule stabilization and organization. Changes in the cleavage, phosphorylation or aggregation of tau may directly lead to the disruption of microtubules architecture. To study the functional effects of Aβ on tau, we used a transgenic *Drosophila* model that expresses fluorescence GFP labeled tau (TauGFP) in subperineural glia cell. TauGFP labeled microtubule fibers are visible throughout the cytoplasm, emerging from the microtubule organizing center (MTOC) adjacent to the nucleus and extending to the cell cortex (Figure 4). Soluble

Aβ1-40 or Aβ1-42 (100pM or 10nM) was microinjected into the body cavity of late embryo (AEL-21-22 hr). Four days later (3rd instar larvae), the central nerve cord was dissected out, mounted in PBS and imaged directly with a confocal microscope [12]. The microtubule cytoskeleton was easily visualized under confocal microscopy which allows fluorescence allowing us to measure changes of microtubule architecture as a result of the Aβ treatment. At 100pM, the microtubule organization of subperineural glia cell injected with Aβ1-42 was destabilized and clustered, while the microtubule architecture was normal in Aβ1-40 injected flies (Figure 4). When we increased the concentration of Aβ to 10nM, a compromised cytoskeleton was observed in both Aβ1-42 and Aβ1-40 injected flies.

Aβ1-42 increases phosphorylated and insoluble tau, when compared with Aβ1-40, in the entorhinal cortex of P301S mice

To further examine the effect of Aβ on tau pathology in vivo, we performed intracerebral injections of Aβ into P301S mice (tau transgenic mouse model) at 8-months of age. At 8-months P301S mice have already developed



tau pathology that increases as the animal age [13]. We targeted the entorhinal cortex as our injection site because neurons in the entorhinal cortex are more sensitive to Aβ-induced stress [14]. Unilateral injection of Aβ1-40 (2ug) into the left entorhinal cortex and Aβ1-42 (2ug) into the right entorhinal cortex allowed us to directly compare the effects of Aβ1-40 and Aβ1-42 in the same mouse.

Eighteen days after injection the mice were perfused with PBS and the brains were isolated, followed by fixation with 4% PFA. As shown in Figure 5A, significant increases in p-tau (recognized by PHF-1 and pS262) and Tau 421 (recognized by tau-C3) were observed in S1 fraction from Aβ1-42- injected entorhinal cortex compared to the Aβ1-40 injected entorhinal cortex. Western blot of P1 fraction from the brain extract (Figure 5C) showed that, a ≥4 fold increase in HT7, ≥6 fold increase

in PHF1 and ≥4 fold increase in pS262 signal in Aβ1-42 treated as compared to Aβ1-40 treated entorhinal cortex. Immunohistochemistry with AT180, which recognizes phosphor-Thr²³¹, suggests that injection of Aβ1-42 promotes greater phosphorylation and aggregation of tau when compared to Aβ1-40 in both the entorhinal cortex and hippocampus (Figure 6).

Aβ1-42 promotes tau pathology while Aβ1-40 may inhibit tau pathology

The difference between Aβ1-40 and Aβ1-42-induced related tau pathology, shown in Figure 5, could be due to either an accelerating effect by Aβ1-42 or an inhibitory effect of Aβ1-40. To address this question, two groups of P301S mice were injected in the entorhinal cortex. One group was injected with PBS into the left entorhinal cortex and Aβ1-40 (2ug) into the right entorhinal cortex,

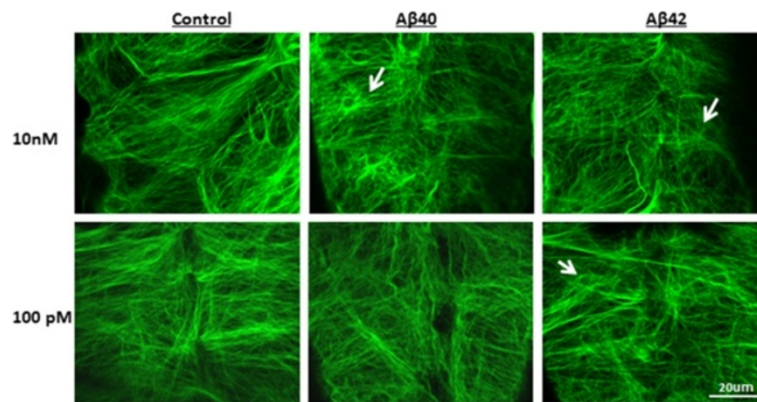


Figure 4 A β 1-42 disrupts cytoskeleton in transgenic fly at 100pM. A β 1-40 or A β 1-42 was diluted in 4% BSA and injected into the body cavity of late embryo of GFP-tau (bovine Tau) transgenic fly (AEL-21-22 hr). 4 days later, the central nerve cord was dissected out, and mounted in PBS and imaged directly with a confocal microscope. Stacks of 20–40 0.5 μ m confocal sections were generated. The results for each section were assembled as a stack. At 100pM, damage to the structure of cytoskeleton was observed in A β 1-42 treated flies but not in flies receiving the A β 1-40 treatment. At 10nM, both A β 1-40 and A β 1-42 led to disruption of the cytoskeleton.

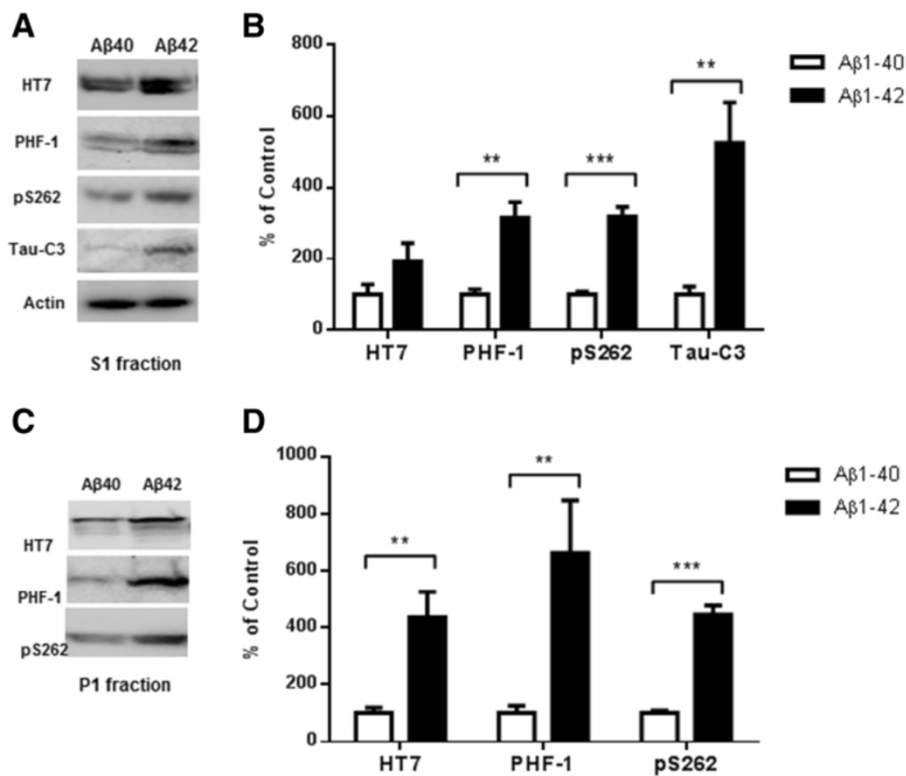


Figure 5 Higher level of total tau, phospho-tau and cleaved tau in P301S mice receiving an A β 1-42 injection into the entorhinal cortex as compared with A β 1-40 injection. 8 month old P301S mice were injected with A β 1-40 into the left entorhinal cortex and A β 1-42 into the right entorhinal cortex. 18 days after injection, the brain extracts were analyzed by western blot. **A**) Representative images of western blots of soluble tau (S1 fraction) detected with anti-tau antibodies and normalized to actin. **B**) Quantification of western blots in **(A)**. **C**) Representative images of western blots of insoluble tau (P1 fraction) detected with anti-tau antibodies. Both S1 brain fraction and P1 brain fraction show a significant increase in HT7, PHF-1 and pS262 signal in entorhinal cortex injected with A β 1-42 compared with A β 1-40 injected entorhinal cortex. Also more cleaved tau (Tau421) was detected in the S1 fraction from the A β 1-42 injected brain. **D**) Quantification of western blots in **(C)**. Histograms show mean \pm standard error, n =3. *p <0.05; **p <0.01; ***p <0.005.

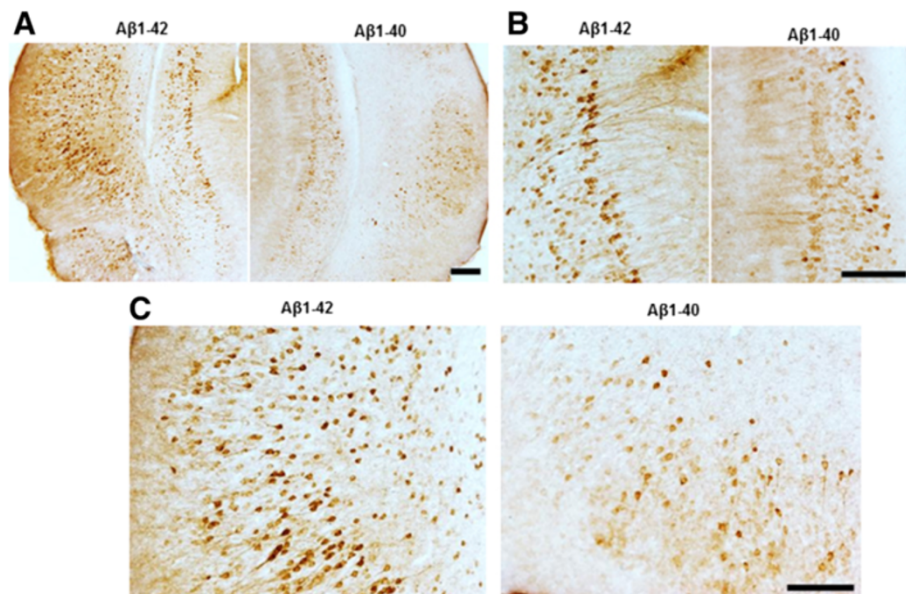


Figure 6 Aβ1-42 treatment increases pathological tau recognized by AT180 when compared with Aβ1-40 treatment. Brain sections were immunostained with AT180, which recognizes p-tau at Thr²³¹. (A) Representative images for pathological tau detected with AT180. (B) Higher magnification showed significantly increased tau tangles in Aβ1-42 injected entorhinal cortex (C) and hippocampus (B). Magnification: 4x for A; 10x for B and C. Scale bar: 100μm.

a second group was injected with PBS into the left entorhinal cortex and Aβ1-42 (2μg) into the right entorhinal cortex. Eighteen days after injection, the mice were sacrificed and the whole hemibrains were dissected and analyzed by western blot. As shown in Figure 7A, there were higher levels of p-tau at Ser²⁶², Ser³⁹⁶ and Ser⁴⁰⁴ in the Aβ1-42- injected side when compared to the contralateral PBS side (S1 fraction in Figure 7A and P1 fraction in Figure 7B). There were also higher levels of cleaved tau and insoluble tau after Aβ1-42 injection. We therefore conclude that Aβ1-42 accelerated tau pathology in P301S mice. For the Aβ1-40 treated group, we didn't observe any differences in total tau, p-tau (PHF-1 specific) or cleaved tau. Interestingly, in the Aβ1-40-injected entorhinal cortex, we observed less phosphorylation at Ser²⁶² in both S1 and P1 fraction.

Discussion

Our data demonstrates that Aβ1-40 and Aβ1-42 have distinct effects on tau phosphorylation, aggregation and cleavage as shown in a variety of in vitro and in vivo models. Most cellular models previously used to study tau pathogenesis used a MTBR (microtubule binding domains) to fragment instead of full-length tau. In recent years, increasing evidence has revealed that self-aggregation of tau into filaments is inhibited by the presence of intact N- and C-termini. The N-terminus is required for the binding of tau to the plasma membrane while cleavage at Asp421 (20 amino acids from C-terminus) significantly increases tau aggregation. Therefore, using full-length tau

(2N4R, 441 amino acids) may be more relevant for studying the metabolism of tau, especially tau cleavage. The construct we used in our experiments, Tau441-YFP incorporates YFP to the C-terminus of tau which doesn't interfere with the normal functions of the N-terminus of tau [15].

Tau is a highly soluble protein and is less prone to aggregate in many cell-based models [16]. To our knowledge, our study is the first to show that full-length tau can aggregate in certain cell type, SHSY5Y and C17.2 cells, as early as 24 h after transient transfection and without the addition of exogenous seeds. In addition, the rate of tau aggregation was not correlated with the level of tau expression because no aggregates formed in N2a cells at 24 h even though our N2a cells expressed a much higher level of full-length tau. For cells with lower degradation efficiency, tau aggregation may occur earlier, consistent with what we found in SHSY5Y and C17.2 cells. Even for cells with high degradation efficiency, continuous overexpression of tau may eventually overwhelm degradation ultimately leading to aggregation. Tau aggregates appeared by 72 h in the N2a cells and stained positively with Thioflavin-S, indicating that overexpression of tau is another factor that contributes to tau aggregation and subsequent fibrilization.

Among the many tau phosphorylation sites, Ser²⁶² is the major site implicated in the abnormal functioning of tau in the AD brain [10,16]. Our biochemical data, from treating stably transfected cell lines overexpressing Tau441-YFP with Aβ1-42, indicate increased phosphorylation at Ser²⁶² in both Triton and SDS soluble

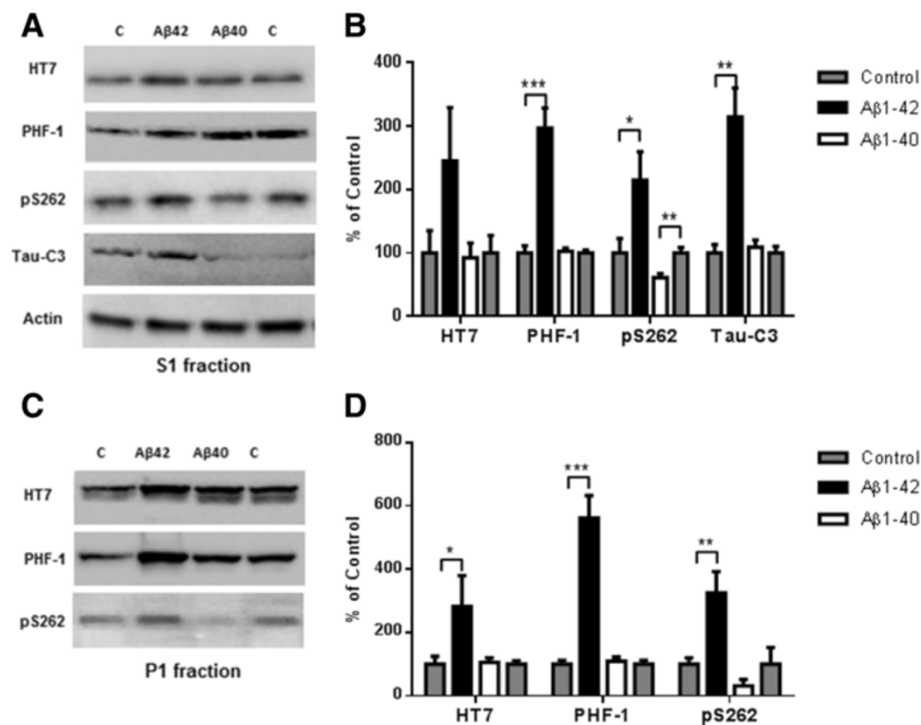


Figure 7 Aβ1-42 promotes tau phosphorylation, cleavage and aggregation while Aβ1-40 does not. 8 month old P301S mice (1N4R form with Prion promoter) were injected with saline into the left entorhinal cortex and Aβ1-42 (2ug) into the right entorhinal cortex or saline into the left entorhinal cortex and Aβ1-40 (2ug) into the right entorhinal cortex. 18 days after injection, the brain extracts were analyzed by western blot. **A**) Representative images of western blots of soluble tau (S1 fraction) detected with anti-tau antibodies and normalized to actin. **B**) Quantification of western blots in **(A)**. **C**) Representative images of western blots of insoluble tau (P1 fraction), detected with anti-tau antibodies. **D**) Quantification of western blots in **(C)** Aβ1-42 injection significantly induced tau phosphorylation (PHF-1 and pS262 epitope) in the S1 and P1 brain fractions. 3-fold increase in Tau421 was found in the soluble fraction (**A and B**). Significant decrease in pS262 immunoreactivity was observed in S1 fraction from Aβ1-40 injected brain areas compared to control side. Histograms show mean +/- standard error, n = 3. *p < 0.05; **p < 0.01; ***p < 0.005.

fractions when compared to cells treated with Aβ1-40 and non-treated cells. Phosphorylation at Ser²⁶² dramatically reduces the ability of tau to bind to microtubules, which ultimately results in microtubule destabilization. Microtubule destabilization affects synaptic vesicle transport and may disrupt synaptic function in vivo [17]. In transgenic *Drosophila* expressing both human Aβ1-42 and tau, Aβ1-42 specifically increases tau phosphorylation at Ser²⁶² and enhances tau-induced neurodegeneration [18]. Co-expression of Aβ1-42 and tau carrying the non-phosphorylatable Ser262Ala mutation did not cause neurodegeneration, suggesting that the Ser²⁶² phosphorylation is required for the pathogenic interaction between Aβ1-42 and tau [18]. We found that at low concentration, Aβ1-40 did not induce phosphorylation at Ser²⁶² in either Triton or SDS soluble fractions in our cell model. This may explain why we do not see an increase in insoluble tau with Aβ1-40 treatment (Figure 2).

Aβ1-42 has been shown to induce tau phosphorylation and the formation of neurofibrillary tangles via a GSK-3β-dependent mechanism in both in vitro and in vivo studies [1,19,20]. Aβ42-induced neurotoxicity seems to

depend on considerable levels of tau that can be inhibited by treatment with GSK-3β inhibitors [21]. The activity of GSK3β is also regulated positively by the phosphorylation of Tyr²¹⁶. Up-regulation of GSK3β activity increases phosphorylation of tau at multiple sites and inhibition of GSK3β specifically decreases the phosphorylation of Ser²⁶² [22]. We found that Aβ1-42 activated GSK3β, but Aβ1-40 did not, which may explain the different effects of Aβ1-40 and Aβ1-42 on tau phosphorylation shown in Figure 3A and C. Our observation of increased Tau421 in Aβ1-42 treated cells, as well as previous studies on the correlation between caspase-3 activation and tau cleavage, indicate that the effects we observed following Aβ1-42 treatment on tau may be mediated by caspase-3 activation. In fact, western blot using a specific antibody to active caspase-3 indicated that Aβ1-42 activates pro-caspase-3 but no activation was observed with Aβ1-40 treatment. This may explain the marked difference we observed between the level of Tau421 in Aβ1-42 and Aβ1-40 treated cells (Figure 3).

In order to find out how Aβ directly affects the structure and function of tau in vivo, we used a tau-GFP

transgenic *Drosophila* model. This is an ideal model because GFP-tagged tau can be used as a subcellular marker to study cytoskeletal dynamics in living cells. The latter avoids potential fixation artifacts and allows for the dynamic visualization of changes in the structure of the cytoskeleton. Another advantage of this model is that low concentrations of A β and short incubation times are sufficient to see the effects of A β on tau. A β 1-42 injections at concentrations as low as 100pM caused cytoskeleton disruption in this model, but we did not see an effect on the cytoskeleton after exposure to low concentration of A β 1-40. Interestingly, when we increased the concentration of A β to 10nM, we saw disrupted cytoskeletal structure in both A β 1-40 and A β 1-42-injected flies. Nonetheless, our data clearly show marked differential effects of A β 1-42 and A β 1-40 on tau function using live cell imaging in this tau transgenic fly model.

In vivo studies using a transgenic mouse model expressing P301S tau were carried out to further assess how the two main A β species may affect tau pathology in a vertebrate system. In AD, the formation of NFTs starts in the entorhinal cortex and spreads to the hippocampus and eventually to most cortical areas [23]. There are some advantages of injection of A β in the entorhinal cortex over other brain areas. First, unilateral injections can be carried out on both the ipsilateral and contralateral entorhinal cortex since there is no cross-talk between the left and right entorhinal cortex [24] which allows for a direct comparison of A β species in the same animal. Second, this approach allows us to study how A β and tau interact at the early stage of the disease. Third, expression of tau in entorhinal cortex can spread along anatomically connected networks to hippocampus [25]. Eighteen days after injection, biochemical analyses with soluble and insoluble fraction from injected brain tissues demonstrated that A β 1-42 promotes tau cleavage, phosphorylation and aggregation. By contrast, treatment with A β 1-40 showed no effect on the total tau or p-tau (recognized by PHF-1) level. Immunostaining with AT180 showed higher levels of p-tau (Thr²³¹) in the entorhinal cortex (injection site), as well as in hippocampus, suggesting that pathological tau induced by A β 1-42 in the entorhinal cortex may spread to other brain areas as reported by Gotz and coworkers [1]. The decrease observed in tau phosphorylation at Ser²⁶² in the presence of A β 1-40 suggests that A β 1-40 may inhibit tau pathology in old tau transgenic mice that already have tangles. It will worth to try breed BRI-A β 40 or BRI-A β 42 mice with tau transgenic mice to see how A β 1-40 or A β 1-42 affects tau pathogenesis in vivo. Also we are working on how combination of A β 1-40 and A β 1-42 changes tau pathology compares to A β 1-42 and study if A β 1-40 inhibits tau pathology induced by A β 1-42.

In conclusion, data from cell-based models, tau-GFP transgenic *Drosophila* and tau transgenic mouse models indicate that A β 1-42 is clearly the pathogenic A β species with respect to inducing the “pathology”; i.e., increased cleavage, phosphorylation and aggregation of soluble wild-type tau. By contrast, A β 1-40 has little to no effect on the pathogenesis. In fact, A β 1-40 may even subserve a protective role in vivo as indicated by the decrease in phosphorylation at Ser²⁶². Therapeutic strategies that preferentially target A β 1-40 thereby increasing the ratio of A β 1-42 to A β 1-40 may actually be problematic. However, targeted A β treatments that lower the ratio of A β 1-42 to A β 1-40 may be of therapeutic benefit.

Methods

A β Preparation: A β ₁₋₄₂ and A β ₁₋₄₀

Dry peptide (1 mg) was pretreated with neat trifluoroacetic acid (1 mL), distilled under nitrogen, washed with 1,1,1,3,3,3-hexafluoro-2-propanol (1 mL), distilled under nitrogen, then dissolved in DMSO to 10 mM, and stored at -20°C [26].

Cell culture and treatment

C17.2 cells (mouse neural progenitor cell line), a gift from Marc Diamond, were grown in DMEM, supplemented with 10% fetal bovine serum, 5% horse serum, and 1% pen/strep. SH-SY5Y cells (human neuroblastoma cell line) were cultured in DMEM/F12 medium supplemented with 10% FBS. N2a cells were grown in DMEM/OPTI-MEM supplemented with 5% FBS.

For transient transfections, cells were plated in 8-well Lab-Tek chamber slides (Nunc) and were transfected using Lipofectamine 2000 constructs (Invitrogen) according to the manufacturer's recommendations.

For treatment: 200nM A β 1-42 or A β 1-40 was added to the medium and imaged at varied time points thereafter.

Live cell imaging

A Live-cell imaging was performed on a Leica TCS SP5 spectral confocal microscope using an oil-immersion 63 \times lens. Observation of the cells with 514-nm laser was performed at low light/laser intensities to prevent phototoxicity.

Staining

For Thioflavin-S staining, cells were incubated with 0.025% Thioflavin-S (in 50% ethanol) for 5 min, rinsed with 50% ethanol and water, and cover slipped for imaging.

For insoluble tau: Cells fixed with 4% paraformaldehyde containing 1% Triton X-100 for 15 min to remove soluble proteins and fluorescence after extraction was recognized as insoluble tau. Fluorescence intensity was quantified with ImageJ.

Sequential extraction and western blot

For generation of stable tau expressing cell lines, individual clones of N2a cells were selected in the presence of 500 µg/ml Geneticin after transfection, then picked and propagated in serum-DME supplemented with 200 µg/ml Geneticin.

N2a cells stably expressing tau-YFP were treated with 200nM Aβ1-42 or Aβ1-40. 24 h later, cells were collected and cell pellets were resuspended in 1% Triton lysis buffer and incubated on ice for 15 min. Following sonication, lysates were centrifuged at 12,000 × rpm for 20 min at 4°C. Supernatants were kept as “Triton fraction,” whereas pellets were resuspended, and sonicated in 1% SDS lysis buffer. After centrifugation at 12,000 × rpm for 20 min at room temperature, supernatants were saved as “SDS fraction.” Equal proportions of Triton and SDS fractions were resolved on 4–20% Tris-glycine midi gel, transferred to nitrocellulose membrane using the iBlot Dry Blotting System, and probed with specific antibodies in Table 1.

Tau-transgenic fly

UAS-tau-GFP transgenic fly line (tau-GFP is driven by SPG-specific moody-Gal4 driver) was used for injection and live imaging. 100 pm or 10nM Aβ1-42 or Aβ1-40 was injected into the body cavity of late embryo (AEL-21-22 hr), and they were allowed to developed until 3rd instar larvae (about 4 days). Then the central nerve cord was dissected out, and mounted in PBS and imaged directly. All confocal images were acquired using a Zeiss LSM 710 system. Stacks of 20–40 0.5 µm confocal sections were generated. The results for each section were assembled as a stack.

Stereotaxic injections of Aβ into entorhinal cortex

P301S mice were purchased from Jackson Labs. All experiments were approved by the Institutional Animal Care and Use Committee at Weill-Cornell Medical College. P301S mice (Jackson labs) were anesthetized with isoflurane and placed in a Stoelting stereotaxic instrument

(Stoelting Co., Wood Dale, Illinois) and an incision was made along the midline. The coordinates for injection of Aβ42 were determined with reference to the bregma at position: AP, -3.6 mm; L, ±3.8 mm; DV, -4.5 mm. Using a 5 µl Hamilton syringe (Hamilton Inc., Bonaduz, Switzerland) driven by a mini pump (Motorized Stereotaxic Injector, Stoelting), a total volume of 2 µl of the Aβ1-42, Aβ1-40 or Saline was injected with an injection speed of 0.25 µl/min unilaterally into the entorhinal cortex. The needle was kept in the injection site for 5 min and then slowly withdrawn to prevent a backflow of the Aβ preparation.

Biochemical analysis for brain extract

Brain tissues were weighed and homogenized in 10 volumes of homogenization buffer (Tris-buffered saline (TBS), pH 7.4, containing 1× protease and phosphatase inhibitor mixture with 2 mM EGTA). The homogenized samples were spun at 21,000 × g for 20 min, the supernatants were centrifuged at 100,000 × g for 1 h at 4°C to obtain insoluble pellet and supernatant (S1 fraction). The insoluble pellet (P1 fraction) was resuspended in 1% sarkosyl in H buffer (10 mM Tris-HCl, 1 mM EGTA, 0.8 M NaCl, 10% sucrose, and protease inhibitor mixture, pH 7.4) and sonicated. The solution was clear and label as P1, diluted in H2O for ELISA [27]. S1 or P1 fraction was separated on 4–20% Tris-glycine midi gel, transferred to iblot gel nitrocellulose using the iblot Dry Blotting System, and probed with HT7, PHF-1, pS262 and tau-C3.

Immunohistochemistry

Brains were placed in 10% buffered formalin, followed by 30% sucrose. These sections were subsequently stained using phosphor-tau antibody- AT180. Secondary antibody was applied, and slides were then incubated with avidin-biotin complex reagent for 5 min. After rinsing, slides were treated with the chromogen 3,3'-diaminobenzidine (Vector Laboratories, SK-4100) to allow visualization.

Table 1 Antibodies

Antibody	Epitope	Source
HT7	Tau (159-161aa)	Pierce (cat#MN1000)
pS262	p-Tau (phosphorylated at Ser262)	Invitrogen (cat# 44-750G)
AT180	p-Tau (phosphorylated at Ser231)	Pierce (cat#MN1040)
PHF-1	p-Tau (phosphorylated at Ser396 and 404)	Generous gift from Peter Davies
Tau-C3	Cleaved tau (cleaved at Asp421)	Millipore (cat#MAB5430)
Anti-caspase-3	Cleaved caspase-3	Cell signaling (cat#9662S)
Anti-GSK-3β	Total GSK3β	BD Sciences (610201)
Anti-GSK3β (phospho Y216)	p-GSK3β (phosphorylated at Tyr216)	Abcam (cat#ab75745)

Abbreviations

AD: Alzheimer's disease; A β : Amyloid beta; NFT: Neurofibrillary tangles.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

XL performed transgenic fly experiment and participated in experimental design and discussion. MZ performed in vivo injection experiment and participated in experimental design and discussion. AG helped with in vivo injection and editing. WL participated in experiment design and discussion. XH conceived and supervised the entire project, performed live cell imaging, western blot and cell culture, also did all of the data analysis, and wrote the manuscript. SP supervised the entire project. All authors read and approved the final manuscript.

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