

RESEARCH ARTICLE

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Age-dependent changes in TDP-43 levels in a mouse model of Alzheimer disease are linked to A β oligomers accumulation

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Abstract

Background: Transactive response DNA-binding protein 43 (TDP-43) is the pathological protein found in frontotemporal lobar degeneration with ubiquitin positive inclusions and in amyotrophic lateral sclerosis. In diseased tissue, TDP-43 translocates from its physiological nuclear location into the cytoplasm, where it accumulates. Additionally, C-terminal fragments of TDP-43 accumulate in affected brain regions and are sufficient to cause TDP-43 mislocalization and cytoplasmic accumulation *in vitro*. TDP-43 also accumulates in 30% of Alzheimer disease (AD) cases, a finding that has been highly reproducible. The role of TDP-43 in AD and its relation with A β and tau pathology, the two neuropathological hallmarks of AD, remains to be elucidated.

Results: Here we show that levels of TDP-43 and its ~35 kDa C-terminal fragment are significantly increased in the 3xTg-AD mice, an animal model of AD that develops an age-dependent cognitive decline linked to the accumulation of A β and tau. We also report that the levels of TDP-43 and its C-terminal fragment correlate with the levels of soluble A β oligomers, which play a key role in AD pathogenesis. Notably, genetically reducing A β ₄₂ production restores the levels of TDP-43 and its ~35 kDa C-terminal fragment to control levels.

Conclusions: These data suggest a possible relation between A β oligomers and TDP-43.

Background

Alzheimer disease (AD) is the most common cause of dementia among the elderly [1]. Clinical symptoms include memory loss and impairments in other domains that interfere with mood, reason, judgment, and language [2-4]. Two hallmark neuropathological lesions of AD include the aberrant accumulation of the amyloid- β peptide (A β) and neurofibrillary tangles (NFTs) [5]. Other prominent changes include intraneuronal A β accumulation, mitochondrial dysfunction, oxidative damage, and changes in the protein quality system [6-9]. A β is the primary protein component of amyloid plaques and originates via proteolysis from the amyloid precursor protein [10,11]. A β has been the central focal point of AD research for more than a decade and is generally considered the upstream causative factor for AD [12]. The strongest evidence for this position is

derived from molecular genetic studies of the three genes (amyloid precursor protein, presenilin 1, and presenilin 2) that underlie familial AD cases, as they all modulate some aspect of A β metabolism, increasing the propensity of A β to aggregate [13-16]. Indeed, A β is an aggregation-prone peptide, and it exists in different forms such as monomers, oligomers, and fibrils [17,18]. In the past few years, *in vitro* and *in vivo* studies have shown soluble A β oligomers to be the major neurotoxic species for neurons [19].

The major component of NFTs is the microtubule-associated protein, tau [20-23]. In its normal state, tau is a soluble protein whose function is to promote microtubule assembly and stabilization. Pathological tau protein, by contrast, exhibits altered solubility properties, forms filamentous structures, and is abnormally phosphorylated at specific residues [20-23]. Recent evidence indicates that the accumulation of soluble, phosphorylated tau may be more toxic than NFTs [24-27].

Transactive response DNA-binding protein 43 (TDP-43) is a nuclear protein involved in exon skipping and

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alternative splicing [28]. The full length fragment has an approximate molecular weight of ~44 kDa. Recently, TDP-43 has been found to be the main protein that accumulates in frontotemporal lobar degeneration with ubiquitin positive inclusions (FTLD-U) and in amyotrophic lateral sclerosis (ALS) [29]. Pathological TDP-43 is mislocalized from the nucleus to the cytoplasm where it accumulates [29]. Additionally, TDP-43 C-terminal fragments have been isolated from affected brain regions [29,30], and their expression *in vitro* is sufficient to cause TDP-43 mislocalization [31-33], suggesting that these fragments may play a role in the disease pathogenesis.

In addition to ALS and FTLD-U, TDP-43 positive inclusions are present in Parkinson disease, dementia with Lewy bodies, and in 30% of AD cases [34-37]. The specific role of TDP-43 in AD has not been identified yet; specifically, it is not clear whether there is a link between TDP-43, A β and tau pathology. In this study, we address the relation between A β , tau and TDP-43 in the 3 \times Tg-AD mice, an animal model of AD that develops A β and tau pathology, with a temporal- and regional-specific profile that closely mimics their development in the human AD brain [38].

Results

The accumulation of TDP-43 has been linked to FTLD-U and ALS [29]. TDP-43 also accumulates in Parkinson's disease, dementia with Lewy bodies, and in 30% of AD cases [34-37,39]. In AD brains, TDP-43 seems to prevalently accumulate in the brain areas more susceptible to A β and tau pathology such as hippocampus, amygdala, and in selected cortical regions [40]. However, the relation between TDP-43, A β and tau is not known. To investigate the link between A β , tau and TDP-43, we first measured the steady-state levels of TDP-43 and its C-terminal fragments in the low salt fraction (see material and methods) of proteins extracted from in 2-, 6-, and 12-month-old 3 \times Tg-AD and NonTg mice (n = 6/genotype/time-point). We found that in the brains of 2-month-old mice, the steady-state levels of TDP-43 and the ~35 kDa C-terminal fragment (herein referred to as TDP-35), which can be detected by overexposing the blots, were similar between 3 \times Tg-AD and age- and gender-matched non transgenic (NonTg) mice (Figure 1A-B). In contrast, the brains of 6-month-old 3 \times Tg-AD mice had significantly higher levels of TDP-43 and TDP-35 than the age- and gender-matched NonTg mice (Figure 1C-D). TDP-43 and TDP-35 levels were again similar between 3 \times Tg-AD and NonTg mice at 12 months of age (Figure 1E-F). The higher levels of TDP-43 and TDP-35 in 6-month-old 3 \times Tg-AD mice coincided with the onset of A β and tau pathology [38,41]. The difference in TDP-43 levels between 3 \times Tg-AD and

NonTg mice at 6 months of age might be due to a decrease in TDP-43 levels in the brains of 6-month-old 3 \times Tg-AD mice or to an increase in TDP-43 levels in 6-month-old NonTg mice. To discriminate between these two possibilities, protein extracted from 2-, 6-, and 12-month-old NonTg mice were run on the same blot. We found that in NonTg mice, the levels of TDP-43 and TDP-35 were similar across the three different time points (Figure 2A-B). In contrast, our results indicated that in the 3 \times Tg-AD mice the levels of TDP-43 and TDP-35 were significantly higher at 6 months of age, compared to 2 and 12 months of age (Figure 2C-D). Taken together, these data indicate that the higher levels of TDP-43 and TDP-35 in the brains of 6-month-old 3 \times Tg-AD mice compared to NonTg mice are due to an increase of these proteins in the 3 \times Tg-AD mice as a function of age and not to a decrease of the levels of TDP-43 and TDP-35 in the brains of NonTg mice.

A neuropathological feature of TDP-43 proteinopathies is the mislocalization of TDP-43, from its predominantly nuclear location into the cytosol, where it accumulates [29]. To determine whether there is mislocalization of TDP-43 and TDP-35 in the brains of the 3 \times Tg-AD mice, we extracted nuclear and cytosolic proteins from the brains of 2-, 6-, 12-month-old 3 \times Tg-AD and NonTg mice (n = 6/genotype/time-point). Using Western blot analysis, we found that at 2 and 12 months of age, the levels of TDP-43 and TDP-35 were not statistically different between 3 \times Tg-AD and NonTg mice in the nuclear and cytosolic fractions (Figure 3A-C, G-I). In contrast, at 6 months of age, the levels of cytosolic TDP-43 and TDP-35 were significantly higher in the 3 \times Tg-AD mice compared to age- and gender-matched NonTg mice (Figure 3D-F). Unexpectedly, we found that the nuclear levels of TDP-43 and TDP-35 were not statistically different between NonTg and 3 \times Tg-AD mice (Figure 3D-F), suggesting that the increase of the levels in the cytosolic fraction is not simply due to a redistribution of TDP-43 from the nucleus into the cytoplasm. As expected, the levels of TDP-43 and its C-terminal fragments were constantly higher in the nuclear fraction compared to the cytosolic fraction (Figure 3A, D, G). Moreover, in the cytosolic fraction, at all ages and independent of the genotype, the predominant species was TDP-35. In the nuclear fraction there were bands with different molecular weights, indicating more TDP-43 species. Future studies are needed to understand the nature of these fragments.

Tau pathology is another hallmark feature of AD and develops in the 3 \times Tg-AD mice as a function of age [38,42]. In the 3 \times Tg-AD mice tau first accumulates in the somatodendritic compartment, and as the mice age, it becomes hyperphosphorylated and eventually aggregates to form NFTs [38,42]. Phosphorylation at Thr181

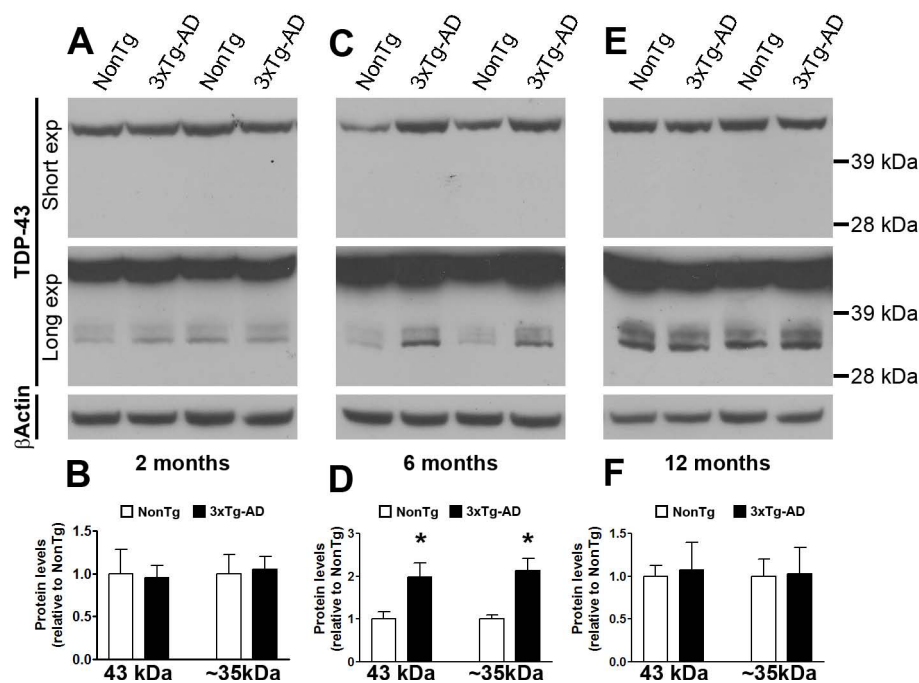
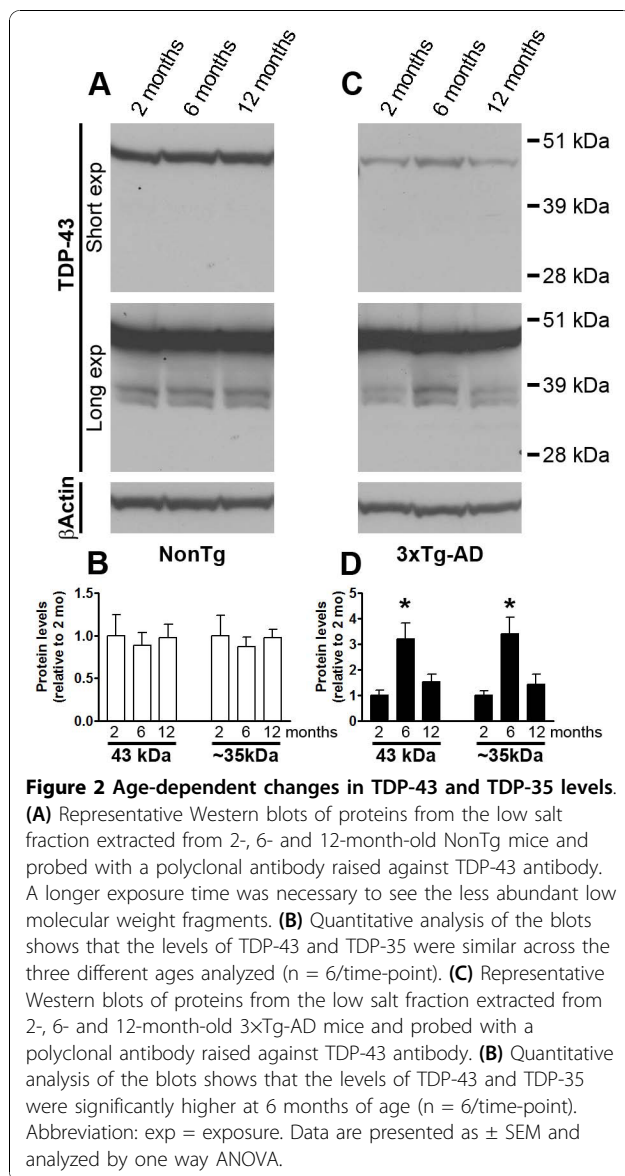


Figure 1 3xTg-AD mice have higher levels of TDP-43 and TDP-35. **(A)** Representative Western blots of proteins from the low salt fraction extracted from 2-month-old 3xTg-AD and NonTg mice and probed with the indicated antibodies. A longer exposure time was necessary to see the less abundant low molecular weight fragments. **(B)** Quantitative analysis of the blots shows that at 2 months of age the levels of TDP-43 and TDP-35 were similar between 3xTg-AD and NonTg mice (n = 6/genotype). **(C)** Representative Western blots of proteins from the low salt fraction extracted from 6-month-old 3xTg-AD and NonTg mice. **(D)** Quantitative analysis of the blots shows that at 6 months of age the levels of TDP-43 and TDP-35 were significantly increased (p = 0.04 and p = 0.01, respectively) in the brains of the 3xTg-AD mice compared to age- and gender-matched NonTg mice (n = 6/genotype). **(E)** Representative Western blots of proteins from the low salt fraction extracted from 12-month-old 3xTg-AD and NonTg mice (n = 6/genotype). **(F)** Quantitative analysis of the blots shows that at 12 months of age the levels of TDP-43 and TDP-35 were similar between 3xTg-AD and NonTg mice (n = 6/genotype). Abbreviation: exp = exposure. Data are presented as ± SEM and analyzed by t-test analysis.

(recognized by the anti-tau antibody, AT270) is one of the earliest manifestations of tau pathology in the 3xTg-AD mice and occurs at 6 months of age (Figure 4A). As the mice age, AT270 immunoreactivity increases (Figure 4A), and the age-dependent increase is also detected by Western blot (Figure 4B-C). We next sought to determine whether there is a relation between AT270, TDP-43 and TDP-35. We focused on the AT270 epitope because in the 3xTg-AD mice tau phosphorylation at Thr181 correlates with cognitive performance (e.g., [43]). Linear regression analysis indicated that the levels of AT270 in the brains of 6-month-old 3xTg-AD mice do not correlate with the levels of TDP-43 and TDP-35 (Figure 4D-E; $r^2 = 0.09148$, $p = 0.56$; and $r^2 = 0.1491$, $p = 0.45$, respectively). Neither was a correlation between AT270 and TDP-43 and TDP-35 levels observed at 12 months of age (Figure 4F-G; $r^2 = 0.4644$, $p = 0.14$; and $r^2 = 0.4469$, $p = 0.15$). The lack of correlation between TDP-43 and tau is consistent with data showing that TDP-43 deposits in AD brains do not colocalize with NFTs or dystrophic neuritis [37].

Evidence indicates a primary role for A β oligomers in AD pathogenesis [12]. In the 3xTg-AD mice, A β oligomerization starts intraneuronally, and a strong immunoreactivity can be detected with the M71/3 antibody, which is specific for low molecular weight A β oligomers (Figure 5A, C; [44,45]). As the mice age, however, the intraneuronal M71/3 immunoreactivity decreases (Figure 5A-D). We have previously shown that this decrease in M71/3 in 12-month-old 3xTg-AD mice correlates with the appearance of extracellular A β plaques [44]. Next, to further analyze the changes in A β oligomer levels in the brains of the 3xTg-AD mice, we performed dot blot experiments using A11, an oligomeric-specific antibody that recognizes high molecular weight A β oligomers [46]. Consistent with the M71/3 data, we found that the A11 levels peak at 6 months of age and decrease at 12 months of age (Figure 5E-F). To determine the relation between TDP-43, TDP-35 and A β oligomers, we correlated oligomeric A β levels obtained by A11 dot blot with TDP-43 and TDP-35 levels in 6- and 12-month-old 3xTg-AD mice. We found that in



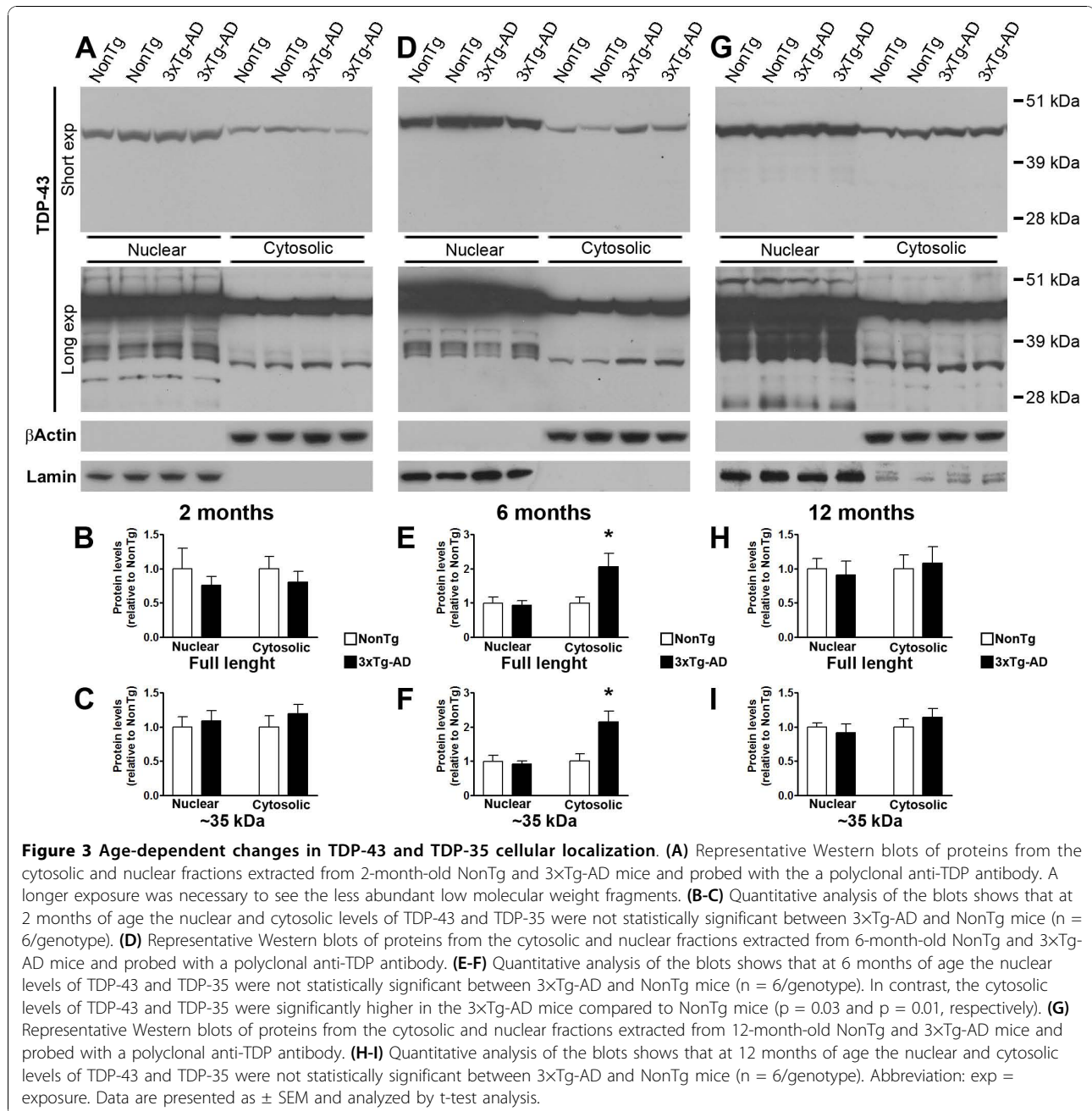
6-month-old 3xTg-AD mice, A11-positive A β oligomers positively correlated with TDP-43 ($r^2 = 0.9330$, $p = 0.001$; Figure 5G) and TDP-35 ($r^2 = 0.6749$, $p = 0.045$; Figure 5H). Similarly, we found that A11 levels in 12-month-old 3xTg-AD mice positively correlate with TDP-43 ($r^2 = 0.7259$, $p = 0.031$; Figure 5I). A strong trend was also observed when analyzing A11 and TDP-35 levels in 12-month-old 3xTg-AD mice ($r^2 = 0.6277$, $p = 0.06$; Figure 5J). Taken together, these data suggest that the increase in TDP-43 in the 3xTg-AD mice may be due to the increase in soluble A β oligomers.

The strong correlation between A β oligomers and TDP-43 levels led us to hypothesize that the buildup of A β oligomers maybe the cause underlying the increase in TDP-43 levels in the 3xTg-AD mice. To test this

hypothesis we used a double transgenic mouse model (referred to as APP/tau mice) that we previously generated by replacing the mutant PS1 allele with its wild type counterpart in the 3xTg-AD mice, thereby obtaining double transgenic mice expressing APP and tau [47]. Because of the M146V mutation in the PS1 gene, the 3xTg-AD mice accumulate 10 times more A β_{42} than A β_{40} [38]. Thus, replacing the M146V mutation with its wild type counterpart significantly decreased A β_{42} levels [47]. Here we used the APP/tau mice to determine the effects of preventing A β oligomers accumulation on TDP-43 levels. At 6 month of age, the APP/tau mice show a significant reduction in intraneuronal A β immunoreactivity compared to age- and gender-matched 3xTg-AD mice (Figure 6A, B). Notably, the APP/tau mice did not show any M71/3 immunoreactivity (Figure 6C). To determine the effect of preventing A β accumulation on TDP-43, we measured the steady-state levels of TDP-43 and TDP-35 in the low salt fraction of proteins extracted from the brains of the APP/tau mice by Western blot (Figure 6D). We found that the levels of TDP-43 and TDP-35 were significantly lower in the brains of the APP/tau mice compared to 3xTg-AD mice (Figure 6E-F). Notably, the levels of TDP-43 and TDP-35 were not significantly different between APP/tau and NonTg mice. Taken together, the results presented here strongly argue of a causal relation between the build-up of A β oligomers and the increase in TDP-43 levels.

Discussion

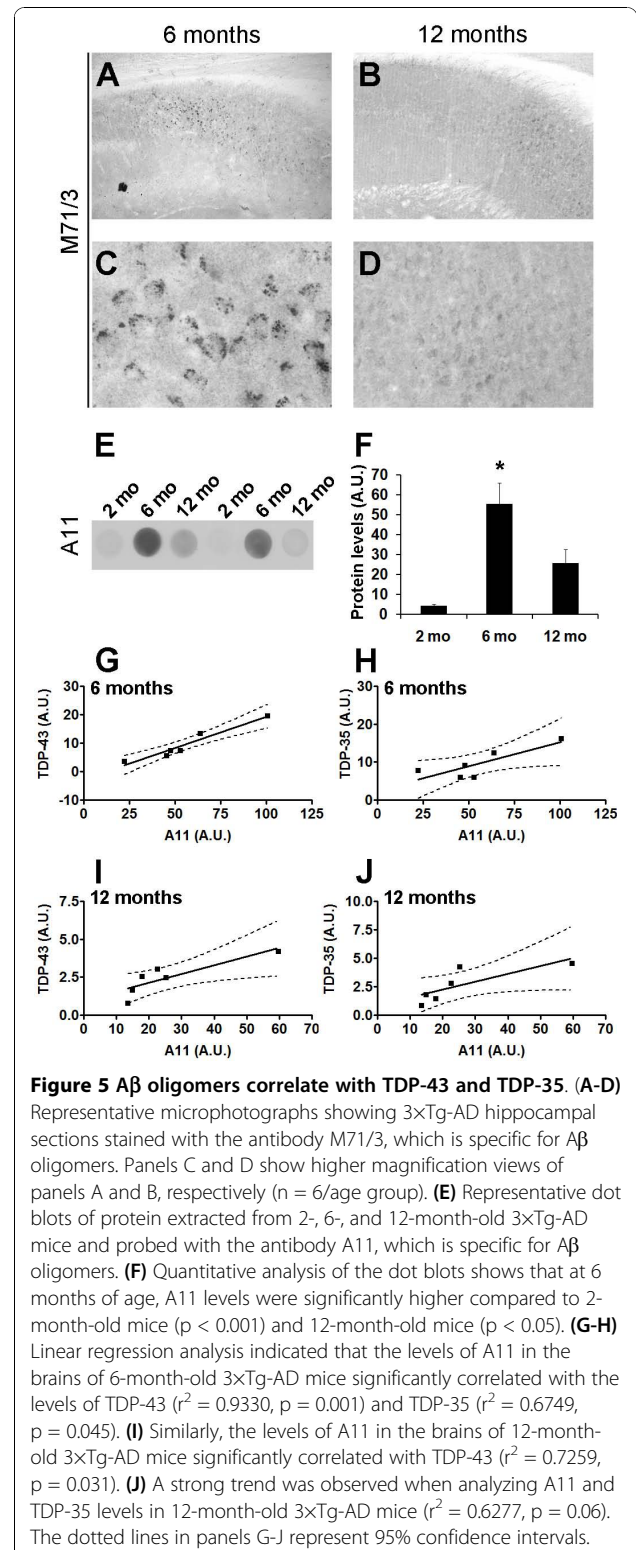
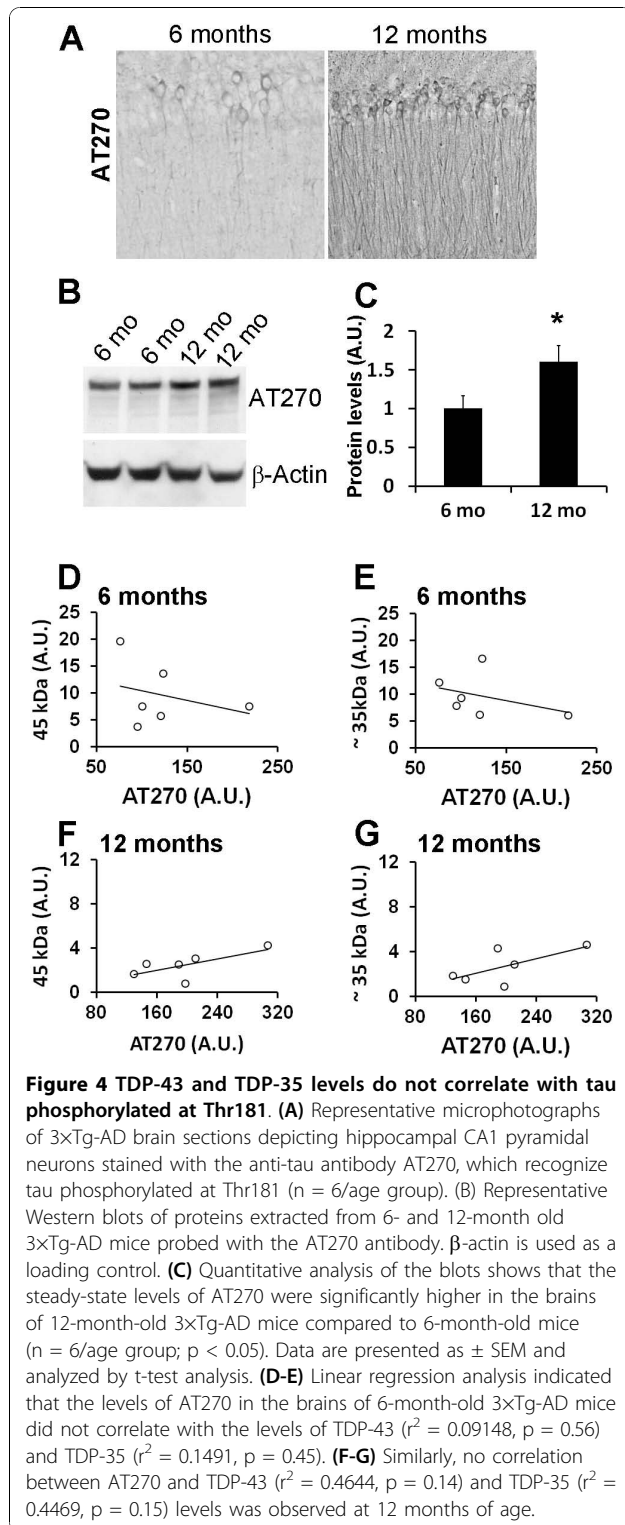
In addition to representing the major pathological protein that accumulates in CNS inclusions characterizing ALS and FTLD-U, TDP-43 positive inclusions have been found in ~30% of AD cases [34,37,39]. Specifically, the accumulation of low molecular weight C-terminal fragments has been reported in human AD patients [48]. Notably, these fragments may play a primary role in the disease pathogenesis as their expression in vitro is sufficient to cause TDP-43 mislocalization [31-33]. The clinical significance of TDP-43 accumulation in AD and its relation with the two neuropathological hallmarks of AD (A β and tau) is not understood. In this study, we elucidate this relation using an animal model of AD. Our results indicate that in the brains of the 3xTg-AD mice the levels of full length TDP-43 and its ~35 kDa C-terminal fragment change as a function of age and A β oligomer levels. Notably, we found that TDP-43 and TDP-35 levels significantly correlated with A β oligomers, thereby suggesting a possible relation between A β and TDP-43. Toward this end, we found that TDP-43 and TDP-35 levels were higher in 6-month-old 3xTg-AD mice compared to age-matched NonTg mice, but not at 12 months of age. Previously we showed that A β oligomers levels in the 3xTg-AD mice peak at 6 months



of age and are significantly lower at 12 months of age [44], which is consistent with the hypothesis that the increased TDP-43 levels in 6-month-old 3xTg-AD mice may be due to high levels of A β oligomers. Indeed, we show that genetically preventing A β ₄₂ accumulation in the 3xTg-AD mice is sufficient to decrease TDP-43 levels, further supporting an interaction between A β and TDP-43.

It is widely accepted that A β oligomers play a central role in AD pathogenesis [17-19]. Toward this end, it has been shown that low concentrations of A β oligomers

can kill neurons, impair LTP, and lead to cognitive decline [49-55]. A β oligomers have been shown to interact with several signaling transduction pathways [56-61]. Although the mechanism underlying the A β -mediated accumulation of TDP-43 in the 3xTg-AD mice remains to be elucidated, it is tempting to speculate that alterations in signaling transduction pathways due to the build-up of A β oligomers may be responsible for TDP-43 accumulation and misprocessing. For example, caspase 3 and 7 can cleave TDP-43 and lead to the accumulation of TDP-43 fragments [62]. Notably,



elevated caspase 7 mRNA levels have been reported in AD brains [63], and, more specifically, there is evidence that $A\beta$ oligomers can increase caspase activity [64-66]. This is consistent with data showing that TDP-43 can

be cleaved, in a caspase dependent manner, to generate TDP-43 C-terminal fragments [62]. Thus, it is tempting to speculate that an $A\beta$ -increase in caspase activity may facilitate the formation of the $\sim 35\text{kDa}$ C-terminal

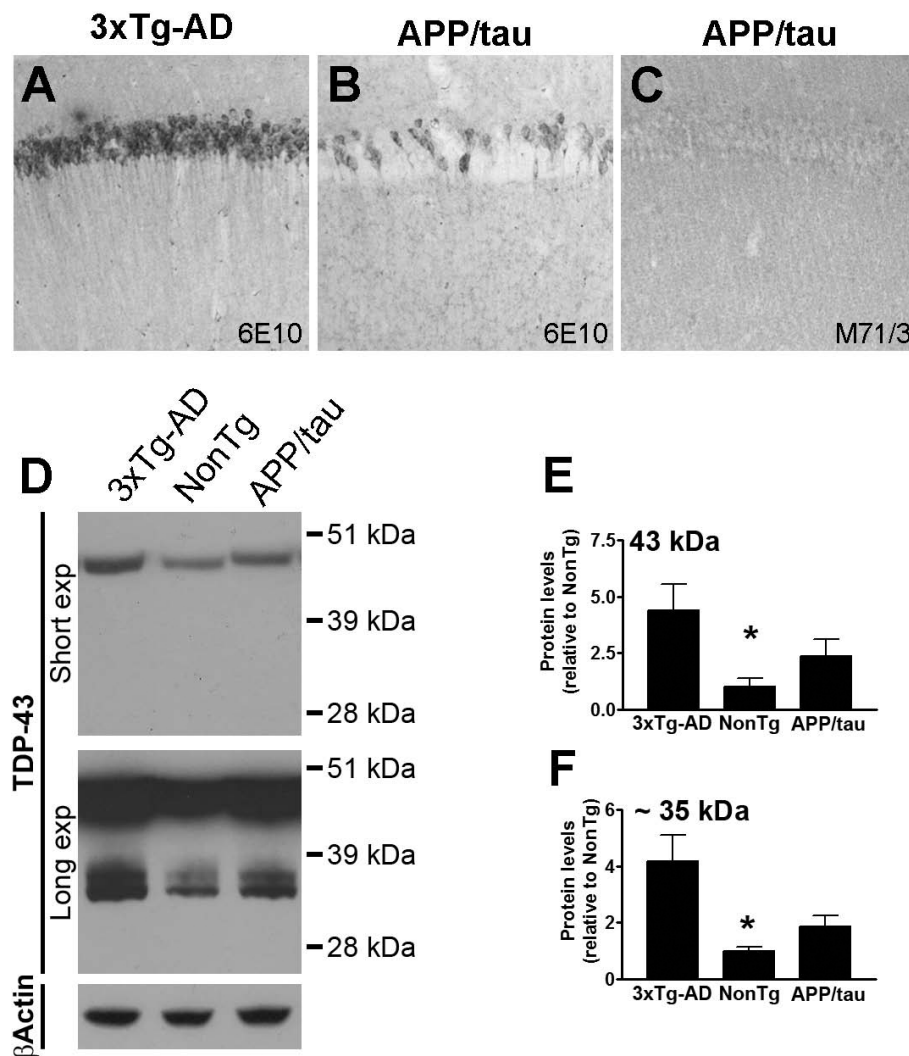


Figure 6 Reduction of Aβ₄₂ levels decreases TDP-43 and TDP-35 levels. (A-B) Representative microphotographs depicting CA1 pyramidal neurons from 6-month-old 3xTg-AD and APP/tau mice stained with an anti-Aβ antibody (n = 6/group). (C) Representative microphotograph depicting CA1 pyramidal neurons from APP/tau mice stained with the anti-oligomeric antibody M17/3. (D) Representative Western blots of protein extracted from the brains of 6-month-old NonTg, APP/tau and 3xTg-AD mice (low salt fraction), probed with a polyclonal anti-TDP-43 antibody. β-Actin is used as a loading control. A longer exposure time was necessary to see the less abundant low molecular weight fragments. (E-F) Quantitative analysis of the blots (n = 6/genotype) shows that in APP/tau mice, the steady-state levels of TDP-43 and TDP-35 are significantly lower in APP/tau mice compared to 3xTg-AD mice (p < 0.05). The levels of TDP-43 and TDP-35 were not statistically significant different between APP/tau and NonTg mice. Data are presented as means ± SEM and were analyzed using one-way ANOVA following by post hoc Bonferroni test to determine individual differences in groups. Abbreviation: exp = exposure.

fragment of TDP-43. Additionally, our results show that cytosolic TDP-43 levels are higher in 6-month-old 3xTg-AD mice compared to age- and gender-matched NonTg mice. Such an increase, however, was not due to a redistribution of TDP-43 from the nucleus into the cytoplasm as we found that nuclear TDP-43 levels were similar between 3xTg-AD and NonTg mice. It is possible that a reduction in protein turnover may account for the higher levels of TDP-43 and TDP-35 in the 3xTg-AD mice. Notably, Aβ oligomers have been shown to

reduce the activity of the ubiquitin-proteasome-system and autophagy [61,67], two major protein turnover systems that are involved in TDP-43 clearance as independently reported by several laboratories [31,68-70].

Contradicting reports have been published on the relation between TDP-43 and tau pathology. Specifically, in brains from AD patients, more often than not, tau immunoreactivity does not correlate with TDP-43 positive neurons [48], which is consistent with our data showing that TDP-43 and TDP-35 levels did not

correlate with phosphorylated tau at Thr181. However, it has been reported that the Braak score for neurofibrillary tau pathology is higher in AD cases with TDP-43 immunoreactivity [48]. To complicate this apparent contradiction are the data showing that in dementia with Lewy bodies, a disease also characterized by tau accumulation, TDP-43 immunoreactivity is not related with Braak neurofibrillary tau pathology [48]. Although we found that in the 3×Tg-AD mice, TDP-43 levels did not correlate with tau phosphorylated at Thr181, further studies are needed to establish whether TDP-43 levels will change in relation to NFT, as suggested by some studies with human brains [34,48], or with total tau levels. Specifically, the latter could not be addressed using the 3×Tg-AD mice, where the tau transgene does not change as a function of age and its steady-state level reflect the promoter activity. Thus any attempt of correlating total tau to TDP-43 would have been artificial. Furthermore, a report by Amador-Ortiz and colleagues shows that in some selective regions of AD brains, TDP-43 deposits correlate with phosphotau, whereas in other regions within the same brain, no correlation was found [34]. This apparent discrepancy could arise from the sample preparation; indeed, the data presented here from the 3×Tg-AD mice where obtained from whole brain homogenize and compare the levels of soluble tau and soluble TDP-43 and TDP-35. Finally, the relationship between other phosphotau epitopes and TDP-43 remains to be determined.

Conclusions

The data presented here provide compelling evidence that in the brain of the 3×Tg-AD mice, the accumulation of soluble A β oligomers may be responsible for the increase in the steady-state levels of TDP-43 and TDP-35. It should be noted, however, that the biochemical profile of TDP-43 detected in the 3×Tg-AD mice was different from that believed pathogenic in FTL-DU [29], suggesting that in these mice changes in TDP-43 levels do not play a role in the AD-like phenotype developed by these mice. Further studies will be needed to elucidate whether TDP-43 plays a clinical role in AD pathogenesis.

Materials and methods

Mice

The generation of the 3×Tg-AD and APP/tau mice was previously described [38,47]. Briefly, the 3×Tg-AD mice were generated by co-injecting two different transgenes encoding human APP^{swe} and human tau P301L, both under the control of the Thy1.2 promoter, into single-cell embryos harvested from homozygous mutant PS1M146V knock-in mice (PS1-KI). The APP/tau mice were generated crossing homozygous 3×Tg-AD mice

with NonTg mice to replace the mutant PS1 allele with its wild type counterpart.

Immunohistochemistry

Mice were sacrificed by CO₂ asphyxiation and their brains rapidly removed and dropped fixed for 48 hours in 4% paraformaldehyde. Free-floating sections (50 μ m thick) were obtained using a vibratome slicing system (Leica VT1200S, Germany) and stored in 0.02% sodium azide in PBS. Following two washes with TBS, endogenous peroxidase activity was quenched for 30 minutes in 3% H₂O₂. For epitope exposure sections were next incubated in 90% formic acid for 7 minutes, followed by three additional washes in TBS (100 mM Tris pH 7.5; 150 mM NaCl). The proper primary antibody was applied overnight at 4°C. Sections were washed 3 times in TBS and then incubated with the suitable secondary antibody for 1 hour at room temperature. Sections were then developed with diaminobenzidine (DAB) substrate using the avidin-biotin horseradish peroxidase system (Vector Labs, Burlingame, CA).

Protein extraction

Following CO₂ asphyxiation, brains were extracted and frozen in dry ice. To obtain the low and high salt fractions, brains were homogenized with a power homogenizer in 1 ml of low salt buffer (10 mM Tris pH7.5, 5 mM EDTA, 1 mM DTT, 10% Sucrose) in the presence of protease inhibitors. Samples were then centrifuged at 14,400 rpm for 30 minutes at 4°C. The supernatant was stored at -80°C as low salt fraction. To obtain the cytosolic and nuclear fractions, brains were washed in PBS and then homogenized with a dounce homogenizer with 2 ml of solution A (10 mM Hepes pH7.9, 10 mM KCL, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT) in the presence of protease inhibitors. After 5 initial strokes, 0.5% of NP40 was added, and the brain was further homogenized with 5 additional strokes. Subsequently, the solution was kept in ice for 10 minutes and centrifuged 1 minute at 11,000 rpm. The supernatant was removed and stored at -80°C as cytosolic fraction. The pellet was re-suspended in 250 μ l of Solution B (20 mM Hepes pH7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) in the presence of protease inhibitors and placed in ice for 15 minutes. Finally, the tubes were centrifuged 5 minutes at 11,000 rpm and the supernatant was stored at -80°C as nuclear fraction.

Western blot and dot blot

Proteins were resolved using precast SDS/PAGE gels (Invitrogen, Carlsbad, CA) under reducing conditions and transferred to a nitrocellulose membrane. The membrane was incubated in a 5% solution of non-fat dry milk in T-TBS (0.02% Tween 20, 100 mM Tris pH

7.5; 150 nM NaCl) for 1 hour at 20°C. The membrane was then incubated in the proper primary antibody at 4°C overnight. The blots were washed in T-TBS for 20 minutes and incubated at 20°C with the appropriate secondary antibody for 1 hour. After a final 20-minute wash in T-TBS, blots were developed for 5 minutes with Super Signal (Pierce, Rockford, IL), washed and exposed. For dot-blots, proteins were applied in a nitrocellulose membrane and air dried. Membranes were resolved as described above.

Antibodies

The following antibodies were used in this study: AT270 (Pierce, Rockford, IL) anti- β -actin (Sigma, St. Louis, MO), rabbit anti human TARDBP polyclonal antibody (ProteinTech Group, Chicago, IL), A11 (a gift from Dr. Charles Glabe, University of California, Irvine), M71/3 (a gift from Dr. William Klein, Northwestern University, Evanston, IL).

Statistical evaluation

The data were subsequently analyzed by ANOVA or t-test comparison as detailed in the figure legends, using Graphpad Prism software (Graphpad Prism Inc., San Diego, CA).

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Authors' contributions

AC designed the study, executed most of the experiments and analyzed the data. AM executed some of the Western blots. SO designed the study, analyzed the data and wrote the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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