Published in final edited form as:

Neurobiol Dis. 2017 July ; 103: 154–162. doi:10.1016/j.nbd.2017.04.012.

TDP-43 expression influences amyloid β plaque deposition and tau aggregation

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Abstract

Although the main focus in Alzheimer's disease (AD) has been an investigation of mechanisms causing A β plaque deposition and tau tangle formation, recent studies have shown that phosphorylated TDP-43 pathology is present in up to 50% of sporadic cases. Furthermore, elevated phosphorylated TDP-43 has been associated with more severe AD pathology. Therefore, we hypothesized that TDP-43 may regulate amyloid-beta precursor protein (APP) trafficking and tau phosphorylation/aggregation. In order to examine the role of TDP-43 in AD, we developed a transgenic mouse that overexpresses hippocampal and cortical neuronal TDP-43 in a mouse expressing familial mutations (K595N and M596L) in *APP* and *presenilin 1* (*PSEN1* E9). In our model, increased TDP-43 was related to increased tau aggregation as evidenced by thioflavin S-positive phosphorylated tau, which may implicate TDP-43 expression in pre-tangle formation. In addition, there was increased endosomal/lysosomal localization of APP and reduced A β plaque formation with increased TDP-43. Furthermore, there was decreased calcineurin with elevated TDP-43 expression. Since calcineurin is a phosphatase for TDP-43, the decreased calcineurin expression may be one mechanism leading to an increase in accumulation of diffuse phosphorylated TDP-43 in the hippocampus and cortex. We further show that when TDP-43 is knocked down there is an increase in calcineurin. In our model of selective TDP-43 overexpression in an *APP/PSEN1* background, we show that TDP-43 decreases A β plaque deposition while increasing abnormal tau aggregation. These observations indicate that TDP-43 may play a role in regulating APP trafficking and tau aggregation. Our data suggest that TDP-43 could be a putative target for therapeutic intervention in AD affecting both A β plaque formation and tauopathy.

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Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Keywords

TDP-43; TARDBP; APP; Calcineurin; Alzheimer's disease; APP/PS1; Tau

INTRODUCTION

Alzheimer's disease is the most frequent form of dementia, accounting for 70 to 80% of all cases. The greatest risk factor for AD is aging; an estimated 30 million people are living with AD and over the next 30 years it is projected to increase to over 130 million worldwide (Dolgin, 2016). In addition, one in three seniors dies with AD or another dementing disease and since 2000, deaths from AD have increased 89% (see 2017 Alzheimer's Disease Facts and Figures, Alzheimer's & Dementia 2017). The pathology of AD consists of extracellular amyloid-beta ($A\beta$) plaques and intraneuronal aggregates of the microtubule-associated protein tau (MAPT), which is hyperphosphorylated in neurofibrillary tangles (NFTs) and neuritic plaques (NPs). In *APP/PSEN1* mice, secreted $A\beta$ monomers produced from APP processing disrupt cognitive function, yet there is little or no NFT or NP formation, synaptic loss or neurodegeneration (Abdul et al., 2008; Borchelt et al., 1997; Walsh et al., 2002). Moreover, there are over a hundred transgenic AD models that have been developed exhibiting either $A\beta$ plaque pathogenesis, NFT formation, neuronal loss or cognitive deficits, but only one of which, the APPSwDI/NOS2^{-/-} mouse, induces endogenous tauopathy (Colton et al., 2008; Wilcock et al., 2008).

Although the main focus in AD research has been the investigation of mechanisms associated with $A\beta$ and tau aggregation, recent studies have shown that in up to 50% of late-onset, sporadic cases of AD TDP-43 proteinopathy has been reported as an age-related comorbidity (Amador-Ortiz et al., 2007; Arai et al., 2009; Higashi et al., 2007; Jellinger, 2008; Lippa et al., 2009; Uryu et al., 2008). TDP-43 functions as a heterogeneous nuclear ribonucleoprotein (hnRNP), with two RNA recognition motifs, nuclear localization and export signals, and a glycine-rich domain involved in protein-protein interactions. TDP-43 participates in exon skipping, RNA stability, RNA transport, splicing, translation, micro-RNA processing, and other cellular functions (Buratti et al., 2010; Buratti et al., 2001; Strong et al., 2007; Wang et al., 2004). Similar to tau, pathological TDP-43 becomes hyperphosphorylated and is present largely as neuronal inclusions or, less frequently, in glial cells (Arai et al., 2006; Neumann et al., 2006). Some studies have shown that TDP-43 proteinopathy may contribute to the cognitive impairment seen in AD (Josephs et al., 2014). Calcineurin is a Ca^{2+} /calmodulin-dependent protein phosphatase known to regulate phosphorylation of TDP-43, long-term depression and depotentiation as well as synaptic plasticity and memory (Hodgkiss and Kelly, 1995; Liachko et al., 2016; Mulkey et al., 1994; Zeng et al., 2001). There is evidence that TDP-43 might be functionally involved in APP regulation and tau aggregation. A recent study demonstrated that knocking out TDP-43 in the hippocampus and cortex in an APP background mouse reduced $A\beta$ plaques, accelerated neurodegeneration, and led to cognitive deficits (LaClair et al., 2016). In order to further characterize the role of TDP-43 in AD, we have developed a mouse model that selectively drives expression of human TDP-43 in the hippocampus and cortex (Camk2a-tTA) in an

APP/PSEN1 mouse background. Here we report the biochemical and histological characterization of this mouse model of AD with TDP-43 proteinopathy.

MATERIALS AND METHODS

Mice

Camk2a-tTA (tet-off, Jackson Lab 007004), TetO-hTDP-43 (developed by M. Gitcho and N. Cairns, Washington University School of Medicine, St. Louis, MO) and APP^{swe}/PSEN1 E9 (*APP/PSEN1*) (Jackson Lab, 005864) mice were maintained on a C57BL/6J background (Jackson Laboratory, stock # 000664) for this study) (Hodgkiss and Kelly, 1995; Liachko et al., 2016; Mulkey et al., 1994; Zeng et al., 2001). TetO-hTDP-43 mice were developed by PCR cloning of human TDP-43 cDNA into the pTRE-Tight response plasmid (Clontech Laboratories, 631059) containing a tetracycline-response element (tetO) and linearized for pronuclear injections at the Washington University Transgenic core facility. Transgenic mice utilized the tetracycline-controlled transactivator protein (tTA, Tet-off) under regulatory control of the hippocampal and cortical neuronal-specific calcium-calmodulin-dependent kinase II (Camk2a, Jackson 003010) promoter (Gossen and Bujard, 1992; Lim et al., 2010; Mayford et al., 1996). Founders were genotyped by PCR using the following primers: TDP-43 forward 5'-GCGTGTACGGTGGGAGGCCTATATA-3', TDP-43 reverse 5'-AGCATCTGTCTCATCCATTTTCTTTT-3'; PS1 forward 5'-AATAGAGAACGGCAGGAGCA-3', PS1 reverse 5'-GCCATGAGGGCACTAATCAT-3'; Camk2a forward 5'-CGCTGTGGGGCATTTTACTTTAG-3', Camk2a reverse 5'-CATGTCCAGATCGAAATCGTC-3'. All experiments were approved by and performed according to the ethical guidelines provided by the Animal Care and Use Committees at Delaware State University and at the University of Delaware (Animal Facility, G. Talhman and F. Warren) (Borchelt et al., 1997). The following genotypes were evaluated: wildtype, Camk2a-tTA/tetO-hTDP-43, *APP/PS1*, and Camk2a-tTA/tetO-hTDP-43/*APP/PS1*.

Primary cortical neuron and human embryonic kidney cell culture

Wildtype mice were crossed with *APP/PSEN1* mice to produce E14–E15 embryos genotyped for individual cortical neuronal cultures. After the meninges were removed, the cortices were dissociated with 0.05% trypsin in HBSS for 10 min at 37°C, and then filtered through a 70 µm cell strainer. Cells were plated on poly-D-lysine (PDL)-coated plates at 3×10^5 cells/cm² in CEMEM (10% fetal bovine serum, 10% horse serum, 2mM L-glutamine, and 1% penicillin/streptomycin) for 45 min in a 37°C humidified incubator (5% CO₂). The medium was changed to Neurobasal medium (GIBCO 21103049) with a B27 supplement containing antioxidants (NB plus AO GIBCO, 17504044) to specifically enhance neuronal growth. After 3 days, media was half changed with NB plus AO and then half changed every 2 days (Igaz et al., 2011). Lentivirus was introduced to drive TDP-43 expression (pLenti6.2V5-DEST, DNASU Plasmid Repository, HsCD00329959). Human embryonic kidney (HEK-293T) cells were maintained in 5% CO₂ and grown in Dulbecco's Modified Eagle Medium supplemented with 1% penicillin/streptomycin and 5% fetal bovine serum. Cells were transfected with either empty vector (pLX304) or pTDP-43-v5 using Lipofectamine 3000 (Invitrogen, L3000015) or TDP-43 siRNA (5'-rGrCrA rArUrA rGrArC rArGrU rUrArG rArArA rGrArA rGrUG G-3', 5'-rCrCrA rCrUrU rCrUrU rUrCrU rArArC

rUrGrU rCrUrA rUrUrG rCrUrA-3') and non-targeting control from Trifecta RNAi Kit (Integrated DNA Technologies) using Lipofectamine RNAiMAX (Invitrogen, 13775150) according to the manufacturer's recommendations. Cells were lysed 72 hours after transfection.

Immunohistochemistry, immunofluorescence, and thioflavin S staining

Formalin-fixed, paraffin-embedded 6µm brain sections were dewaxed, hydrated, and microwaved for 3 min in 0.01 M sodium citrate blocked in 5% BSA, 0.3% triton-X in PBS for one hour, and incubated overnight at 4°C with primary antibodies. Standard immunohistochemistry techniques were used as previously described (Gitcho et al., 2009). For thioflavin S staining, paraffin-embedded 6µm sections were incubated with 1% aqueous thioflavin S (Sigma, T1892) for 8 min at room temperature then rinsed with deionized water followed by 3 min in 80% ethanol twice and 3 min in 95% ethanol. Slides were rinsed with 3 changes of deionized water before mounting.

Antibodies

All antibodies used in this study are described in Supplementary Table 1.

Western blot, immunoprecipitation, and proteomics

Western blots were generated using standard protocols as previously (Gitcho et al., 2009). For immunoprecipitation (IP), tissue was lysed in an optimal IP buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 100 µM sodium orthovanadate, and a protease inhibitor cocktail (Sigma, P8340). For proteomics, TDP-43 IPs were run on a 15% SDS-PAGE gel, stained with SimplyBlue (Invitrogen, 46-5034), and then excised and digested with trypsin (proteomic results submitted as Davis et al., 2017, *Journal of Biological Chemistry*). Postmortem human brain cortex, obtained from the Knight Alzheimer's Disease Research Center, Washington University School of Medicine, was homogenized in IP buffer, precleared with Protein G agarose beads at 4°C before IP validation. TDP-43 antibody (C-terminal mouse monoclonal), control mouse and rabbit IgG were cross-linked to magnetic beads according to the manufacturer's protocol and incubated overnight at 4°C (ThermoFisher Scientific, 88805). In order to eliminate non-specific binding, two high salt (50mMTris-HCl pH 7.5, 500mM NaCl, 0.1% NP-40, 0.05% sodium dexoycholate) washes followed by two low salt (50mMTris-HCl pH 7.5, 0.1% NP-40, 0.05% sodium dexoycholate) washes were employed before elution and Western blotting.

Mouse transcriptome exon array

RNA was isolated using TRIZOL reagent (Life Technologies, 15596026) as per the manufacturer's instructions. GeneChip Mouse transcriptome array (Affymetrix; wild-type (n=7), *APP/PS1* (n=7), Camk2a-tTA/TDP-43 (n=6), and Camk2a-tTA/TDP-43/*APP/PS1* (n=7).) was performed under manufactures instructions (Jennifer Holbrook, Biomolecular Core Laboratory at Nemours' Center for Pediatric Research) and results analyzed using Transcriptome Analysis Console v.2.0 (Affymetrix).

cDNA synthesis and Quantitative Real-time PCR

Cortex and hippocampus was extracted from 9-month-old mice and homogenized in Trizol reagent (Invitrogen, 15596026). RNA was isolated according to the manufacturer's protocol. cDNA was synthesized using High Capacity Reverse Transcription Kit (ThermoFisher Scientific, 4368813) with 2 µg of RNA per reaction. Quantitative real-time PCR was performed using Maxima SYBR Green/Fluorescein qPCR Master Mix (ThermoFisher Scientific, k0241) and LightCycler480 Real-time PCR System (Roche) as previously described (Gitcho et al., 2009). The following primers were used: beta-actin forward 5' - *TCCTAGCACCATGAAGATCAAG-3'*, beta-actin reverse 5' - *GAAGGTGGACAGTGAGGC-3'*; APP forward 5' - *TGTGCCAGCCAATACCGAA-3'*, APP reverse 5' - *CCAGAACCTGGTCGAGTGG-3'*; MAPT (isoform A) forward 5' - *GTCGGAGACCTCCGATGC-3'*, MAPT (isoform A) reverse 5' - *CCTTCTGGGATCTCCGTGTG-3'*; MAPT (isoform D) forward 5' - *GGCTTAAAAGCTCGTGTGGC-3'*, MAPT (isoform D) reverse 5' - *GAGGTGTGGCGATCTTCG-3'*, calcineurin forward 5' - *TGCAAAGCGCTACTGTTGAG-3'* and calcineurin reverse 5' - *GAGGTGGCATCCTCTCGTTA-3'*

Statistical analysis

Data analyses were performed using a one or two-way ANOVA with Bonferroni or Tukey correction for type-one error (Prism, GraphPad Software). Data are expressed as mean ± standard deviation (n = 3). Biological replicates and littermate controls were evaluated for all the *in vivo* experiments described.

RESULTS

Mice selectively expressing human TDP-43 in the hippocampus and cortex in an *APP/PSEN1* background show no change in splicing

We developed a mouse model that selectively drives expression of human TDP-43 in the hippocampus and cortex in an *APP/PSI* mouse background. The tetO-TDP-43 transgenic mice were under control of the hippocampal and cortical neuronal-specific calcium-calmodulin-dependent kinase II (*Camk2a*, Jackson 003010) promoter (Fig. 1A) (Gossen and Bujard, 1992; Lim et al., 2010; Mayford et al., 1996). Our mating strategy was to first generate double transgenics of *Camk2a-tTA(+)/APP/PSI(+)*. The *Camk2a-tTA(+)/APP/PSI(+)* mice were crossed with *tetO-hTDP-43(+)* mice and maintained on a doxycycline diet (Bio-Serve S3888, 200mg/kg) to suppress hTDP-43 expression until weaning (P24) in order to produce *Camk2a-tTA(+)/tetO-hTDP-43(+)/APP/PSI(+)* mice (*Camk2a/hTDP-43/APP/PSI*). In 9-month old mice, human TDP-43 (Abnova, 555nm) expression was distributed throughout the hippocampus and cortex (Fig. 1B). Expression of human APP was verified through Western blot with human-specific anti-APP (Covance, 6E10) (Fig. 1C & D). Endogenous expression of mouse TDP-43 was significantly reduced in *Camk2a-tTA(+)/tetOTDP-43(+)* mice (0.39 ± 0.08) and *Camk2a/hTDP-43/APP/PSI* mice (0.43 ± 0.07), however there was an overall 1.45 ± 0.13 fold and 1.49 ± 0.07 fold increase in total TDP-43 expression compared with wild-type and *APP/PSI* mice. *APP/PSI* mice had no significant change in TDP-43 expression (1.028 ± 0.15). In order to investigate whether this modest

change in hTDP-43 expression affects splicing we utilized the Mouse Transcriptome Array and found no significant change in mRNA splicing (wild-type (n=7), APP/PS1 (n=7), Camk2a-tTA/TDP-43 (n=6), and Camk2a-tTA/TDP-43/APP/PS1 (n=7)); Affymetrix GeneChip Mouse transcriptome array table. The total mRNA of some transcripts were significantly increased when comparing WT to *APP/PS1* and WT to *Camk2a/hTDP-43/APP/PS1* however, there was no significant difference in mRNA expression when comparing WT to *Camk2a/hTDP-43* or WT to *Camk2a/hTDP-43/APP/PS1* (Supplementary Table 2). In addition, we showed no significant change in splicing when comparing all the genotypes described above, which may be due the low level of hTDP-43 expression evaluated in this model (splicing data not shown).

TDP-43 inversely regulates calcineurin expression

Since calcineurin is an essential phosphatase associated with long-term depression and depotentiation and plays a role in synaptic plasticity and memory, we wanted to investigate the role overexpression of TDP-43 has on calcineurin expression in cortical neurons from APP/PS1 mice (Hodgkiss and Kelly, 1995; Mulkey et al., 1994; Zeng et al., 2001). In both wildtype and *APP/PS1* primary cortical neurons overexpressing TDP-43, there was a significant decrease in calcineurin expression (WT vs. WT+TDP-43, 0.52 ± 0.09 , $p=0.002$; APP vs. APP+TDP-43, 0.53 ± 0.026 , $p=0.0112$) (Fig. 2A & C). In addition to validating the interaction of TDP-43 and calcineurin, we assayed calcineurin expression in our *Camk2a/hTDP-43* transgenic mouse that overexpresses TDP-43 specifically in neurons in the cortex and hippocampus (Supplementary Fig. 1A). Calcineurin expression was significantly lower in the *Camk2a/hTDP-43* versus the non-transgenic control at four months of age (0.58 ± 0.047 , $n=3$, $p=0.005$) and nine months of age (0.52 ± 0.15 $p=0.0003$) with no change in mRNA expression (Supplementary Fig. 1B–D). In order to determine if TDP-43 affects the expression of calcineurin, human embryonic kidney cells (HEK-293T) were transfected with either a non-targeting control (siNTC) or siTDP-43. Knockdown of TDP-43 resulted in an increase in calcineurin (1.7 ± 0.09 , $p=0.044$) suggesting that TDP-43 negatively regulates calcineurin (Fig. 2B & D).

Calcineurin depletion in a *C. elegans* model of ALS increased phosphorylated TDP-43 and exacerbated motor deficits (Liachko et al., 2016). Furthermore, a yeast hybrid screen identified calcineurin as a binding partner of phosphorylated TDP-43 (S409/410), indicating that the phosphatase calcineurin functionally dephosphorylates pathological TDP-43 (Liachko et al., 2016). This work supports the increase in cytoplasmic phosphorylated TDP-43 observed in our model of hTDP-43 overexpression (Fig. 2E). We also observed phosphorylated TDP-43 in APP/PS1 mice, which is likely due in part to stress causing cytoplasmic TDP-43 localization as seen in a tau mouse model (Ayala et al., 2011a; Clippinger et al., 2013; Herman et al., 2011; Kabuta et al., 2015; Moujalled et al., 2013). (Fig. 2E) It has been shown that hyperphosphorylation occurs post aggregation and may be protective and possibly more soluble (Li et al., 2011). Thus a reduction in calcineurin levels may impede pathogenesis. Although our model of overexpression increased phosphorylated TDP-43 due to changes in calcineurin, in the 9-month old *APP/PS1* model, we observe an increase in endogenous phosphorylated TDP-43 that may be an early protective response to stress (Fig. 2E). Thus, marginal increases in TDP-43 phosphorylation may be initially

protective possibly by causing a reduction of calcineurin. As the accumulation and aggregation of TDP-43 increases with age this may foster a prion-like aggregation (Li et al., 2011; Mompean et al., 2016). TDP-43 has been shown to localize to stress granules prior to forming protein aggregates (Liu-Yesucevitz et al., 2010; Parker et al., 2012) and our data also show that TDP-43 colocalizes with stress granules (TIAR) in mice selectively expressing TDP-43 (Supplementary Fig. 2). This suggests an early accumulation of TIAR/pTDP-43 stress granules that may be initially protective before becoming protein aggregates (Liu-Yesucevitz et al., 2010; Parker et al., 2012). Collectively, these data indicate a relationship between decreased calcineurin expression and increased TDP-43 phosphorylation that may contribute to AD pathology.

Selective expression of TDP-43 in the hippocampus and cortex increases endosomal/lysosomal localization of APP

At 9-months of age, the *Camk2a/hTDP-43/APP/PS1* mice show a significant decrease in A β plaque deposition when compared to the APP/PS1 littermate controls (Fig. 3A & B). A β plaques were counted blindly from hippocampal and cortical sections of three mice per genotype and immunostained with anti-A β 1–16 (6E10) antibody. In APP/PS1 there were 18.7 ± 6.1 plaques and only 5.3 ± 2.5 A β plaques counted in *Camk2a/hTDP-43/APP/PS1* mice ($p=0.025$). Since APP is internalized and processed through the endocytic/lysosomal system and increased neuronal endocytosis is an early pathological event observed in AD, we used several markers of early and late endosomes and lysosomes to evaluate the intracellular accumulation of APP in *Camk2a/hTDP-43/APP/PS1* mice (Li et al., 2011). Early endosome antigen 1 (EEA1) and Rab5 were used as immunocytochemical markers for early endosomes whereas Rab7 and lysosome-associated membrane protein 2A (LAMP2A) are markers of late endosomes and lysosomes, respectively. We found that APP co-localized with endosomes at every stage of the endocytic pathway throughout the cortex of both *APP/PS1* and *Camk2a/hTDP-43/APP/PS1* mice and appeared to accumulate markedly in lysosomes (Fig. 3 & Supplementary Fig. 3). Expression of TDP-43 did not affect expression of APP mRNA (Supplementary Fig. 5). Overexpression of TDP-43 has been shown to induce an increase in autophagy; however, in our model we saw no change in autophagy markers LC3B and ATG12 (Supplementary Fig. 4). These observations may indicate that the marginal increase in TDP-43 expression that we observed was insufficient to induce autophagy (Bose et al., 2011; Ying et al., 2016). Interestingly, endosomal acidification and synaptic vesicle endosomal formation is also tightly linked to calcineurin and might account for the change in APP subcellular trafficking observed in this model.

Phosphorylated tau aggregation in mice expressing TDP-43 resembles AD pathology

In order to evaluate the impact of TDP-43 overexpression on tau phosphorylation in the APP/PS1 mouse, we used immunoblotting to assay changes in phosphorylated tau. We detected a higher molecular weight band of approximately 100kD in mice selectively expressing hTDP-43 alone or hTDP-43 in an APP/PS1 background, which was not detected in wildtype or APP/PS1 littermates (Fig. 4A–C). This 100kD band may indicate aggregation of tau. In *Camk2a/hTDP-43/APP/PS1* mice, neurites display diffuse thioflavin S(+) staining that are immunoreactive for phosphorylated tau (pTauSer396) compared with APP/PS1 mice that were Thioflavin S-negative and pTauSer396-positive which may implicate TDP-43

expression in tau pre-tangle formation (Fig. 4D, **see arrows**). This is similar to what is observed in the AD brain (e.g. an 83-year-old female with AD and Braak NFT stage V) (Fig. 4D). AD cases immunostained with anti-TDP-43 showed a change in localization of endogenous TDP-43 (Fig. 4D). Interestingly, this change in localization did not deplete the nucleus of TDP-43, though the staining in the cytoplasm was thioflavin S-positive (Fig. 4D). We next wanted to investigate whether Tau and TDP-43 interact. Coimmunoprecipitation of Tau and TDP-43 in the cortex and/or hippocampus from APP/PS1 and Camk2a-tTA/TDP-43/APP/PS1 mice showed no interaction between soluble Tau and TDP-43 (Supplementary Fig. 5). Recently, a study described an interaction between TDP-43 and Tau mRNA 3'UTR. When TDP-43 interacts with Tau mRNA expression of Tau is suppressed (Gu et al., 2017). When pathological TDP-43 accumulates in the cytoplasm in this TDP-43 M337V ALS mouse model, Tau expression increases and aggregates (Gu et al., 2017). We next examined mouse Tau mRNA isoforms (NM_001038609 isoform A and NM_001285455 isoform D) and human APP mRNA and found that both were not increased due to changes in TDP-43 expression (Supplementary Fig. 6). These results may indicate an early stage of Tau aggregation associated with TDP-43 expression increasing cytoplasmic phosphorylated TDP-43 accumulation. Which supports the role TDP-43 plays in Tau mRNA metabolism described by Gu and colleagues (Gu et al., 2017).

Total TDP-43 colocalizes with thioflavin S and phosphorylated Tau without nuclear depletion of TDP-43 in AD

There is an ongoing debate on whether TDP-43 neurotoxicity stems from nuclear clearance that results in loss of TDP-43 function or a gain of function in the cytoplasm. Several mouse models depict both situations and resemble phenotypes of ALS, FTD, or AD, and display evidence of mitochondrial dysfunction, neurotoxicity and motor or cognitive impairments (Cataldo et al., 1997). To investigate whether our mouse model of TDP-43 pathology without nuclear depletion correlated with the human disease, we immunostained neuropathologically well-characterized cases of AD (two females aged 83 years and one male aged 84 years) all with Braak NFT (neocortical) stage V (Fig. 5A, B, & C). Although cytoplasmic TDP-43 aggregation is noted (cells positive for TDP-43 and thioflavin-S staining), TDP-43 was not depleted from the nucleus (Fig. 5A, B, & C, **see arrows**). Interestingly, thioflavin S also colocalized with TDP-43 in the cytoplasm in the absence of pTau (Fig. 5A, B, & C, **see arrows**). These data support the hypothesis that TDP-43 aggregation, amyloid-like structures, may propagate in a prion-like dependent manner (Mompean et al., 2016). Furthermore, we observed thioflavin S and pTAUSer396 colocalized with TDP-43 (Fig. 5A–C, **see asterisks**). This change in localization of endogenous TDP-43 in some hippocampal cells may be a response to stress induced by severe A β plaque deposition and tauopathy.

DISCUSSION

TDP-43 pathology has been described in up to 50% of sporadic and 14% of familial AD cases (Amador-Ortiz et al., 2007; Arai et al., 2009; Higashi et al., 2007; Jellinger, 2008; Lippa et al., 2009; Uryu et al., 2008). One study looked at 342 pathologically determined AD cases and 57% had some degree of comorbid TDP-43 proteinopathy (Josephs et al.,

2014). This study also showed that TDP-43 positive subjects were more likely to have cognitive impairment at age of death compared with those without TDP-43 pathology. In order to understand the importance of TDP-43 in AD, we developed a mouse model that selectively drives human TDP-43 expression in the hippocampus and cortex in an APP/PS1 background.

The phosphatase calcineurin regulates TDP-43 phosphorylation (Liachko et al., 2016). We further validated the interaction of TDP-43 and calcineurin and our data show that changes in expression of TDP-43 inversely regulate calcineurin expression. When TDP-43 is overexpressed *in vivo*, we show that calcineurin is down regulated thereby increasing accumulation of phosphorylated TDP-43. In addition, endogenous phosphorylated TDP-43 accumulates in APP/PS1 mice, which correlates with reduced levels of calcineurin. Interestingly, mutant tau transgenic mice also show an accumulation of phosphorylated TDP-43; however, calcineurin levels were not evaluated in that model (Clippinger et al., 2013). Calcineurin protein levels are inversely correlated with dementia severity and Braak stage in the AD brain. Also, when globally normalized, calcineurin levels are reduced in AD brain (Karch et al., 2013). Endosomal acidification and synaptic vesicle endosomal formation is also tightly linked to calcineurin and might account for the change in APP subcellular trafficking observed in our model (Cheung and Cousin, 2013). We further show that overexpression of TDP-43 alone and in an APP/PS1 background increases thioflavin S-positive phosphorylated tau staining and higher molecular weight aggregates of phosphorylated tau. This diffuse staining of thioflavin S may indicate an early stage of aggregation or pre-tangle formation not normally seen in *APP/PS1* mice. As with the ALS model described above, when phosphorylated TDP-43 accumulates in the cytoplasm, Tau mRNA becomes more stable and expression is increased (Gu et al., 2017). Though we show no increase in total Tau or phosphorylated Tau (PHF1 or pTauSer396), we do show aggregation, which suggests a role for TDP-43 in Tau aggregation. To our knowledge, this is the first study to look at phosphorylated TDP-43 pathology in AD associated with calcineurin levels. In future studies, we plan investigate the role of calcineurin in AD with and without comorbid TDP-43 proteinopathy. These studies may shed light on the relationship between the interaction between TDP-43 calcineurin in AD and other TDP-43 proteinopathies.

A recent study described knocking out TDP-43 in the hippocampus and cortex and this was proposed as a model of nuclear clearance of TDP-43, similar to that seen in AD (LaClair et al., 2016). Though this model shows severe neuronal loss due to depletion of TDP-43 from the hippocampus and cortex, it does not address the TDP-43 pathology that we describe in our model. In severe cases of AD, we show that endogenous TDP-43 does not become entirely depleted from the nucleus as described previously. During the formation of TDP-43 cytoplasmic inclusions, nuclear TDP-43 appears to be maintained in some of the neurons near A β plaques (Fig. 5). TDP-43 has been shown to self-regulate expression by binding to its own mRNA in order to maintain homeostasis (Avendano-Vazquez et al., 2012; Ayala et al., 2011b). We propose that the ability of TDP-43 to self-regulate diminishes as it accumulates in the cytoplasm to form inclusion bodies in AD and possibly other TDP-43 proteinopathies. Therefore, endogenous nuclear TDP-43 might be expected to increase expression in order to maintain homeostasis. We hypothesize that in late stages of AD,

accumulation of TDP-43 in the cytoplasm may contribute to regulating APP subcellular localization and tau aggregation by increasing nuclear expression of TDP-43. Collectively, our data lead us to conclude that TDP-43 contributes to calcineurin dysregulation, a reduction in A β plaque formation, tau aggregation, and accumulation of phosphorylated TDP-43. Our current data suggest that TDP-43 could be a putative target for therapeutic intervention in AD by modulating both A β plaque formation and tauopathy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank the participants and their families for donating brain tissue to the Knight Alzheimer's Disease Research Center, Washington University, St. Louis, MO, USA. We acknowledge the excellent technical assistance provided by Benjamin Vincent. We further want to acknowledge the Histochemistry and Tissue Processing Core at Nemours Alfred I. DuPont Hospital for Children with special thanks to Heather Hardy and Brian Owens supported by NIGMS: P20GM103446. In addition we wish to thank Brian Kraemer for his discussions about calcineurin. We wish to thank Diane Chugani of the University of Delaware, M. Shahriar Salamat of the Wisconsin Alzheimer's Disease Research Center Neuropathology Core, and especially Jeffrey Johnson of the School of Pharmacy at the University of Wisconsin-Madison for their continuing support. We thank Jennifer Holbrook and Katina Sol-Church of the Biomolecular Core Laboratory at Nemours' Center for Pediatric Research supported by grants from the National Institutes of Health (NIH) through the National Institute of General Medical Sciences (NIGMS) P20GM103464, P30GM114736, and P20GM103446.

Funding: The following work was supported by a University of Wisconsin Alzheimer's Disease Research Center Pilot Grant, Alzheimer's Association New Investigator Research Grant: NIRG-12-241456, the National Institute on Aging: 1K01AG042500, Delaware IDeA Network of Biomedical Research Excellence (INBRE) Pilot Award: NIH-NIGMS: 5P20GM103446, NIH-NIGMS Centers of Biomedical Research Excellence (COBRE): 5P20GM103653 and a Delaware Economic Development Office Grant from the State of Delaware.

Abbreviations

AD	Alzheimer disease
APP	Amyloid precursor protein
Aβ	A β
CTF	C-terminal fragment
RIPA	Radio-immunoprecipitation assay buffer
ANOVA	Analysis of variance
DAD	Definite Alzheimer's Disease
PHF1	Paired helical filament, Phosphorylated Tau Ser396/404 (provided by Peter Davies)
LAMP2	Lysosome-associated membrane protein 2
EEA1	Early endosomal antigen 1
DAPI	4',6-diamidino-2-phenylindole

APP/PS1	Transgenic mice expressing APP Swedish mutation and PSEN1 with deletion of exon 9
Dox	Doxycycline
tTA	Tetracycline transactivator
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
TDP-43	TAR-DNA-binding protein-43
hTDP-43	Human TDP-43
ELISA	Enzyme-linked immunosorbent assay
WT	Wildtype (non-transgenic)
MAPT	Microtubule-associated protein tau

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HIGHLIGHTS

- Selective overexpression of human TDP-43 in the cortex and hippocampus of a mouse model of Alzheimer disease significantly reduces A β plaque formation and increases APP endosomal/lysosomal localization.
- Phosphatase calcineurin interacts with TDP-43 and is inversely regulated by changes in TDP-43 expression.
- This novel mouse model of TDP-43 overexpression exhibits an increase in phosphorylated TDP-43 and phosphorylated tau similar to that of Alzheimer's disease.

Figure 1. Generation of *Camk2a-tTA/hTDP-43/APP/PS1* mice

A) *Camk2a-tTA* mice were crossed with *APP^{swe/PS1^{de9}}* (*APP/PS1*) mice. *Camk2a-tTA* or *Camk2a-tTA/APP/PS1* mice were then crossed with *TetO* mice containing human *TDP-43* (*tetO-hTDP-43*) and were fed Doxycycline (Dox) to keep hTDP-43 expression off during development. Mice were weaned 22 days after birth and Dox was removed from their diet. Upon removal of Dox, the tTA expressed from the *Camk2a* promoter was free to bind to the tetO promoter, thus allowing expression of the human TDP-43 transgene. **B)** Immunofluorescence staining probed for human-specific TDP-43 (555nm, red, Abnova) and total TDP-43 (488nm, green, ProteinTech Group). **C)** Western blot of 9-month old mice confirms expression of human TDP-43 (Abnova) and APP (6E10) in transgenic mice. There was a significant reduction in endogenous TDP-43 in *Camk2a/hTDP-43* and *Camk2a/hTDP-43/APP/PS1* mice detected with mouse-specific anti-TDP-43 antibody. **D)** Western blot quantitation of normalized endogenous TDP-43. **E)** Western blot quantification of normalized total TDP-43 expression. (*p<0.05, ***p<0.0001).

Figure 2. TDP-43 overexpression reduces calcineurin

A) Western blots for *APP/PS1* and wildtype primary cortical neurons infected with human TDP-43 virus. **B)** Western blot of calcineurin expression in HEK293T cells transfected with either non-targeting siRNA (siNC) or siTDP-43. Knockdown of TDP-43 increased calcineurin expression. **C and D)** Quantitation of normalized calcineurin expression by Western blots in A and B, respectively. * $p < 0.05$, ** $p < 0.001$, One-way ANOVA with Bonferroni multiple comparison correction was performed for A. Student t-tests was performed for B. **E)** Phosphorylated TDP-43(Ser409/410) was more prevalent in Camk2a/

hTDP-43/APP/PS1 mice but also observed in APP/PS1 mice. Arrows indicate cytoplasmic accumulation of diffuse endogenous phosphorylated TDP-43(S409/410) (phospho-TDP, Cosmobio, green; TDP-43, Genscript, red; DAPI, blue) 1000X magnification.

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Figure 3. Selective expression of TDP-43 in the hippocampus and cortex alters A β plaque deposition and localization of APP

A) Sagittal sections from APP/PS1 and Camk2a/hTDP-43/APP/PS1 (littermates) display a distinct change in plaque deposition with both 10D5 (**A**) and 6E10 (**B**). **C)** Total A β plaques were blindly counted throughout the cortex and hippocampus of 3 sections from 3 different mice per genotype and immunostained with 6E10 antibody. Camk2a/hTDP-43/APP/PS1 mice had a significant reduction in the number of A β plaques. **D)** Intracellular levels of APP (6E10, Covance, red, 555nm) co-localized with the early endosomal protein RAB5 (Cell

Signaling, green, 488nm), 1000X magnification. **E)** Representative staining shows an increased co-localization of APP (6E10, red, 555nm) and the late endosomal/lysosomal marker LAMP2 (Cell Signaling, green, 488nm) in Camk2a-tta/hTDP-43/APP/PS1 mice, 1000X magnification. Nuclei, shown in blue, are labeled with DAPI.

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Figure 4. Phosphorylated tau aggregation in mice expressing TDP-43

A) PHF1 (phospho-Tau Ser396/404) Western blot in 9-month-old mice. Asterisk indicates phospho-tau at a higher molecular weight of approximately 100kD. **B**) Quantification of PHF1 and higher molecular weight PHF1 **C**) normalized to total Tau. **D**) Thioflavin S/ pTAU(Ser396)-positive cortical cells in Camk2a/hTDP-43/APP/PS1 mice are similar to what is observed in those with AD. Arrows point to cells that are positive for both thioflavin S staining and pTau(Ser396), 400X, (thioflavin S green; pTAU Ser396, red; and TDP-43, blue). Graphs show mean and standard deviation, n=3, One-way ANOVA and Tukey's correction for multiple comparison, *** indicate p-value<0.0001.

Figure 5. Total TDP-43 colocalizes with thioflavin S and phosphorylated Tau in AD

A, B, and C are hippocampal sections from three separate cases of neuropathologically confirmed AD (Braak NFT stage V). Immunofluorescence staining probed with total TDP-43 (N-terminal, rabbit polyclonal, 647nm, blue), phosphorylated Tau (Ser396) (555nm, red, Cell Signaling), and thioflavin S (488nm, green). Arrows indicate cells with cytoplasmic accumulation of TDP-43 staining positive for thioflavin S and negative for pTau (Ser396). Asterisks indicate thioflavin S positive tangles colocalized with TDP-43 and pTAU(Ser396), 400X magnification.