LETTER

TDP-43 interacts with pathological τ protein in Alzheimer's Disease

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Alzheimer's disease (AD) is the leading cause of dementia in elderly people and is characterized by two major neuropathological hallmarks: amyloid β -protein ($A\beta$)-containing senile plaques and neurofibrillary tangles (NFTs) consisting of abnormal phosphorylated τ protein (p- τ). Besides these proteins, transactive response DNA-binding protein 43 (TDP-43) was also discovered to accumulate in the limbic regions of AD brains, by forming cytoplasmic inclusions [1, 2, 10]. Studies have shown that in AD, TDP-43 is associated with smaller hippocampal volumes and more severe cognitive impairment [7]. Accordingly, patients with abnormal TDP-43 immunoreactivity were shown to have a clinically more severe degree of dementia, compared to those without TDP-43 inclusions [6, 8].

Some studies have shown the co-localization of TDP-43 and p- τ pathology in the AD brain, as well as the presence of TDP-43 in NFTs [3, 12, 13]. Moreover, TDP-43 spreading pattern in the brain reminisces that of p- τ [5]. However, whether these two proteins interact and how they are associated during AD pathogenesis was not yet addressed.

Here, we report the association and interaction of TDP-43 with p- τ in human symptomatic and non-symptomatic AD cases using histochemical and biochemical approaches. We have analyzed 13 non-diseased controls and 10 pathologically-defined pre-clinical AD cases (p-preAD) with TDP-43 pathology, which exhibited AD-related neuropathological changes but lacked AD symptoms during their lifetime. We also included 25 symptomatic AD cases (sympAD) with TDP-43 pathology and used 9 FTLT-TDP cases as positive controls for TDP-43 pathology (Suppl. Tab. 1, online-resource).

To investigate the co-occurrence of pTDP-43 and p- τ in the different morphological TDP-43 lesions, we first performed double-label immunofluorescence with anti-phosphorylated TDP-43 (pTDP-43) and anti-p- τ antibodies (Suppl. Tab. 2, online-resource). We observed that sympAD cases displayed dystrophic neurites positive for both proteins (Fig. 1a). As for neuronal cytoplasmic inclusions (NCIs), we observed some inclusions positive for pTDP-43, but negative for p- τ (Fig. 1a). Further, we observed neurofibrillary tangle-like structures positive for pTDP-43 and p- τ (Fig. 1a).

We then quantified the severity of pTDP-43 lesions (DNs, NCIs or NFT-like material), as well as p- τ pathology (p- τ -positive NFTs) in our cohort. The severity of pTDP-43 pathology was significantly increased in sympAD and FTLT-TDP cases, when compared to non-AD cases (Fig. 1b, Suppl. Tab. 3, online-resource). Additionally, NFT severity was also significantly increased in sympAD cases compared to non-AD and FTLT-TDP cases (Fig. 1c). p-preAD cases showed a trend towards an increased severity compared to non-AD and FTLT-TDP cases (Suppl. Tab. 4, online-resource).

To address whether neurons that present NFTs still contain physiological nuclear TDP-43, we performed co-staining with antibodies against non-phosphorylated N- (N-t, 1-50aa.) or C-terminal (C-t, 260-414aa.) epitopes of TDP-43 and p- τ (Suppl. Tab. 2, online-resource). We observed that most neurons with NFTs or pretangles still have non-phosphorylated TDP-43 in the nucleus (arrows, Suppl. Fig. 1, online-resource). Only few p- τ -positive cells presented a nucleus cleared of TDP-43 (arrowheads in Suppl. Fig. 1, online-resource).

Further, we performed double-labelling with the Gallyas silver staining and anti-pTDP-43 (pS409/pS410) in sympAD cases with TDP-43 pathology. We observed that Gallyas-positive NFTs co-localized with pTDP-43 in most neurons (arrows in Suppl. Fig. 2, online-resource). Interestingly, some Gallyas-positive NFTs did not co-stain with pTDP-43 pathology (asterisks in Suppl. Fig. 2, online-resource). Conversely, few pTDP-43-positive neurons did not co-stain for NFTs (arrowheads in Suppl. Fig. 2, online-resource).

To analyze the extent of the association of these proteins, we quantified their co-localization. We measured the ratio of neurons containing co-localized pTDP-43 and p- τ relative to the total amount of pTDP-43-positive neurons in the CA1 region of the hippocampus. We showed that in p-preAD cases, 70,4% of neuronal pTDP-43 inclusions co-localized with p- τ , whereas in sympAD cases there was 78,3% of co-localization (Fig. 1d, e). When quantifying the co-localization of pTDP-43 and p- τ relative to the total amount of p- τ pathology, we obtained a similar result. p-preAD cases presented 74,7% of co-localization and sympAD cases 61,5% (Suppl. Fig. 3, online-resource). Some neurons presented NFT-like material or NCIs positive only for TDP-43 (Fig. 1d, arrowheads) or for p- τ (Fig. 1d, asterisk). FTLT-DTP cases presented significantly less co-localizations of these proteins considering that these cases present scant τ pathology. In sympAD cases, we detected co-localization of cytoplasmic p- τ with non-phosphorylated C-t TDP-43, but not with N-t TDP-43. (Fig. 1d). Notably, the very few NFTs in FTLT-DTP cases were negative for pTDP-43.

In order to confirm the interaction of pTDP-43 and p- τ in AD, we performed proximity ligation assays (PLA), which detects protein-protein interactions based on physical proximity of the proteins (30-40nm). We observed a pronounced expression in sympAD cases (Fig. 1f), when compared to non-AD and FTLT-DTP cases, exhibiting little to no signal. This confirms that pTDP-43 interacts with p- τ in the AD hippocampus.

Further, we validated this interaction biochemically. We used sarkosyl-insoluble fractions of entorhinal cortex homogenates from non-diseased controls, p-preAD, sympAD and FTLT-DTP cases. First, we observed a significant difference in the ratio of 43kDa TDP-43/45kDa TDP-43 bands between non-diseased and sympAD cases (Fig. 1g, Suppl. Tab. 5, online-resource). This indicates an increase of full-length, phosphorylated 45kDa TDP-43 in sympAD cases in sarkosyl-insoluble fractions. p- τ was evidently abundant in sympAD cases. Non-AD, p-preAD and FTLT-DTP cases showed no p- τ expression in the entorhinal cortex (Fig. 1h). We then immunoprecipitated this fraction with anti-p- τ antibodies. After immunoblotting the eluent with C-t TDP-43, we detected a strong expression of 43kDa and full-length 45kDa TDP-43 in sympAD cases (Fig. 1i), being less prominent in the remaining groups. When performing a semi-quantitative assessment of the ratio between the input 43kDa TDP-43 band and the IP 43kDa TDP-43 band, we observed a significant difference between sympAD and FTLT-DTP cases (Fig. 1k, Suppl. Tab. 6, online-resource). The ratio between the input 45kDa TDP-43 band and the IP 45kDa TDP-43 band also showed an increase in sympAD cases, although this was not significant

(Fig. 11). These data confirm that pTDP-43 as well as physiological TDP-43 interact with pathological τ in sympAD cases.

Taken together, TDP-43 pathology is associated with p- τ in NFTs and pretangles, in the human AD hippocampus. This result confirms findings of other authors [1, 3, 4, 9, 13] and extends them as follows: 1) the co-localization and interaction of these proteins is already present in p-preAD cases; 2) biochemical analysis confirmed the interaction of TDP-43 with p- τ in sympAD cases and showed a relative increase in the amount of full-length 45kDa pTDP-43 over 43kDa TDP-43.

Previously, we have shown different molecular patterns of TDP-43 pathology in AD, from being completely absent to the exhibition of only pTDP-43 (pS409/pS410) in typical AD cases to the full-blown picture of TDP-43 lesions, exhibiting non-phosphorylated and full-length forms of TDP-43 as in FTLD-TDP cases (positive for pS409/pS410 as well as pS403/404) [14]. Other authors have recently shown that FTLD-TDP subtypes can be distinguished with epitope phosphorylation changes [11]. These data argue in favor of the hypothesis that TDP-43 aggregates display distinct strains in different diseases. However, TDP-43 is not essential for developing AD because AD cases without TDP-43 pathology exist [1, 14], but TDP-43 aggregates may still modulate AD pathology and presumably its clinical course [5, 10].

TDP-43 pathology in elderly individuals and AD patients is currently considered to represent a separate limbic-predominant, age-related TDP-43 encephalopathy (LATE) [10]. The interaction of pTDP-43 with p- τ in sympAD and p-preAD shown here, however, supports the hypothesis that pTDP-43 pathology in AD cases is part of the AD spectrum rather than representing a separate disease.

Thus, the question arises whether the LATE spectrum of TDP-43 pathology as currently defined covers different types of TDP-43 proteinopathies, one participating in the AD spectrum and probably another one that is independent from AD [10, 14]. Our findings support the hypothesis of an AD-type of LATE pathology. Further studies comparing genetic risk profiles among AD cases with and without TDP-43 pathology and LATE cases may help to clarify the role of TDP-43 in AD.

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Competing Interests

DRT received speaker honorarium from Novartis Pharma Basel (Switzerland) and Biogen (USA), travel reimbursement from GE-Healthcare (UK), and UCB (Belgium), and collaborated with GE-Healthcare (UK), Novartis Pharma Basel (Switzerland), Probiobdrug (Germany), and Janssen Pharmaceutical Companies (Belgium).

Figure legend

Figure 1 – (a) CA1-hippocampus of a sympAD case (case 33). p- τ (pSer202/pThr205) associates with pTDP-43 (409/410) in different morphological lesions, such as dystrophic neurites and neurofibrillary tangle-like structures. Some neuronal cytoplasmic inclusions are exclusively positive for pTDP-43. Scale bars=25 μ m. **(b)** Semi-quantitative quantification of severity of pTDP-43-positive lesions and **(c)** p- τ -positive NFTs **(c)** in non-AD (n=13), p-preAD (n=10), sympAD (n=25) and FTLD-TDP (n=9) cases. Non-parametric, Kruskal-Wallis test or Wilcoxon Signed Rank test with Bonferroni correction for multiple testing were used, *p<0.05; **p<0.01. It is essential to note that the semiquantitative scores for pTDP-43 and p- τ pathology cannot be directly compared as the maximum pathology observed for each lesion was considered as maximum score (3), respectively. Since p- τ pathology is more abundant than pTDP-43 pathology the scores for p- τ pathology represent more abundant lesions than similar scores for pTDP-43 pathology. **(d)** Immunofluorescence of the CA1-hippocampus of a p-preAD (case 17) and a sympAD case (case 33). p- τ co-localizes with pTDP-43 in the same neuron in p-preAD and sympAD cases (arrows). Some neurons are exclusively positive for pTDP-43 (arrowheads) or for p- τ (asterisk). p- τ co-localizes with non-phosphorylated C-t TDP-43 epitopes, but not N-t TDP epitopes. Scale bars=25 μ m. **(e)** Quantification of co-localization of p- τ and pTDP-43 in the CA1-hippocampus relative to the amount of total pTDP-43 lesions. Five p-preAD, seven sympAD and four FTLD-TDP cases were compared. Non-parametric, Kruskal-Wallis test with Bonferroni correction for multiple testing was used. *p<0.05; **p<0.01. Non-AD cases were not assessed due to the scant amount of TDP-43 and τ lesions to be quantified. **(f)** Proximity Ligation Assay (PLA) with antibodies against p- τ (pSer202/pThr205) and pTDP-43 (409/410) shows interaction between these proteins in the CA1-hippocampus in AD cases. Non-AD (n=3, case 7 shown), sympAD (n=3, case 36 shown) and FTLD-TDP (n=3, case 52 shown). Scale bars=50 μ m. **(g)** C-t TDP-43 and **(h)** p- τ immunoblot of sarkosyl-insoluble extracts from non-AD (n=5), p-preAD (n=5), sympAD (n=8) and FTLD-TDP (n=5) cases were used. Cases used are indicated. Remaining cases are shown in Suppl. Fig.4 **(i)** C-t TDP-43 immunoblot of immunoprecipitated p- τ (pSer202/pThr205) from sarkosyl-insoluble extracts. Non-AD (n=5), p-preAD (n=3), AD (n=6) and FTLD-TDP (n=5) cases were used. Respective IP negative controls are indicated (extract and beads with no antibody). Cases used are indicated. Uncropped blots and remaining cases are shown in Suppl. Fig.4, online-resource. **(j)** Semi-quantitative quantification of the

ratio 43kDa/45kDa TDP-43 band (area under the curve). Non-parametric, Kruskal-Wallis test with Bonferroni correction for multiple testing. **(k)** Semi-quantitative quantification of the ratio of 43kDa/ IP 43kDa TDP-43 band (area under the curve). Non-parametric, Kruskal-Wallis test with Bonferroni correction for multiple testing. **(l)** Semi-quantitative quantification of the ratio of 45kDa/ IP 45kDa TDP-43 band (area under the curve). Non-parametric, Kruskal-Wallis test with Bonferroni correction for multiple testing.

For antibody description see Suppl. Tab. 2, online resource.

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PLA

a

pTDP-43	p- τ	Merge
[Image]	[Image]	[Image]
[Image]	[Image]	[Image]
[Image]	[Image]	[Image]

b

Semi-quantitative score of hippocampal TDP-43 pathology

Group	Score (approx.)
non-AD	0.5
p-preAD	2.0
sympAD	1.9
FTLD-TDP	2.4

c

Semi-quantitative score of hippocampal + NFT pathology

Group	Score (approx.)
non-AD	0.7
p-preAD	1.5
sympAD	2.6
FTLD-TDP	1.0

d

p-preAD

pTDP	p- τ	pTDP/p- τ /DAPI
[Image]	[Image]	[Image]
C-t TDP	p- τ	C-t/p- τ /DAPI
N-t TDP	p- τ	N-t/p- τ /DAPI

e

Neurons positive for pTDP-43 and p- τ relative to all pTDP-43 pathology exhibiting neurons (%)

Group	% (approx.)
p-preAD	70
sympAD	80
FTLD-TDP	5

f

non-AD	sympAD	FTLD-TDP
[Image]	[Image]	[Image]

g

non-AD	p-preAD	sympAD	FTLD-TDP
[Western Blot]	[Western Blot]	[Western Blot]	[Western Blot]

h

neg. ct preAD	neg. ct sympAD	neg. ct FTLD
[Western Blot]	[Western Blot]	[Western Blot]

i

neg. ct preAD	neg. ct sympAD	neg. ct FTLD
[Western Blot]	[Western Blot]	[Western Blot]

j

Ratio: input 43kDa / input 45kDa

Group	Ratio (approx.)
Non-AD	2.5
p-preAD	1.8
sympAD	1.1
FTLD-TDP	2.6

k

Ratio: input 43kDa / IP 43kDa

Group	Ratio (approx.)
Non-AD	0.2
p-preAD	0.2
sympAD	1.5
FTLD-TDP	0.2

l

Ratio: input 45kDa / IP 45kDa

Group	Ratio (approx.)
Non-AD	0.5
p-preAD	1.1
sympAD	1.8
FTLD-TDP	1.0

Online resources:

Supplementary Material to: LETTER

TDP-43 interacts with pathological τ in Alzheimer's Disease

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Materials and Methods

Human cases

A total of 57 cases were used in this study. Thirteen non-Alzheimer's disease (non-AD) cases, 10 pathologically diagnosed preclinical AD (p-preAD) cases with TDP-43 pathology, 25 symptomatic AD (sympAD) cases with TDP-43 pathology and 9 cases with frontotemporal lobar degeneration with TDP-43 pathology (FTLD-TDP) were selected for in this study (Suppl. Table 1). All autopsy brains were received from university or municipal hospitals in Leuven (Belgium) and Offenbach am Main (Germany), and collected in accordance with local ethical committee guidelines and the federal laws governing the use of human tissue for research in Belgium and Germany. Dementia was diagnosed according to the DSM-IV criteria. The neuropathological diagnosis of AD was made when dementia was observed before death and when at least an intermediate degree of AD-related neuropathology was determined according to current criteria for the neuropathological diagnosis of AD as published by the National Institute of Aging and Alzheimer Association working group (NIA-AA criteria) [7]. The degree of dementia was determined retrospectively using the Clinical Dementia Rating (CDR) score [6, 10]. For this purpose, the information from the clinical files was used to provide a CDR score. The CDR score was applied in controls and AD cases with AD clinical symptoms [10], whereas CDR with FTLD modules was used when scoring cases with a clinical picture of FTD [9]. SympAD cases were cases with AD pathology and a CDR score of 1 or higher, whereas p-preAD cases were non-demented, i.e. CDR = 0; one case with a CDR score of 0.5 (case No. 16) was considered as not-yet demented as the mild symptoms were not conclusive for the diagnosis of AD and, thus, classified as p-preAD. Non-AD was considered in the event that the definition of AD neuropathological changes according to the National Institute of Aging and the Alzheimer Association (NIA-AA) [7] was not met, i.e. absence of amyloid plaques. Those cases included cases with primary age-related tauopathy (PART).

SympAD and p-preAD cases without TDP-43 inclusions were not included in this study because the aim of this study was the analysis of the interaction of p- τ with TDP-43.

Immunohistochemistry

The left hemispheres were fixed in formalin for 2 weeks, dissected, dehydrated overnight (O/N), and embedded in paraffin blocks. 5 μ m sections were cut using a microtome and mounted on immunohistochemistry microscope slides (Dako).

Sections of the hippocampal formation were stained with antibodies against TDP-43 and τ . Briefly, after citrate epitope retrieval (pH 6) and deparaffinization (using a Dako autostainer Link 48, Dako, Glostrup, Denmark), the sections were treated with peroxidase blocking reagent (Envision flex Peroxidase-Blocking Reagent, Dako) for 5 minutes. Primary antibodies (Suppl. Table 2) were applied for 30 minutes. Afterwards, the slides were incubated with an appropriate secondary antibody (Envision Dual flex, Dako). 3,3'-diaminobenzidine (Liquid DAB+ Substrate Chromogen System, Dako) was used as a chromogen to yield brown reaction products. Counterstaining with hematoxylin was performed. In sections of the hippocampus, entorhinal, temporal and occipital cortex, A β and p- τ immunostaining was performed using a similar protocol as described above, but with formic acid pre-treatment for antigen retrieval prior to the immunostaining procedure.

For red and brown double label immunohistochemistry (Suppl. Fig. 1), slides were stained in an automated BOND system (Leica Biosystems, IL, US) with DAB chromogen (BOND Polymer Refine Detection, Leica) and subsequently with FastRed (BOND Polymer Refine Red Detection, Leica).

For immunofluorescence purposes, the tissue was processed in a similar manner to the aforementioned procedure. After low pH pre-treatment and de-paraffinization, the slides were incubated with the following antibody cocktails overnight: rabbit pTDP43 + mouse p- τ (AT8), rabbit TDP-43 C-term+mouse p- τ (AT8) or rabbit TDP-43 N-term+ mouse p- τ (AT8). After washing, the slides were incubated with a secondary antibody cocktail (rabbit Cy2 + mouse Cy3). The slides were then incubated with DAPI (1:5000) and mounted with ProLong Gold Antifade Mountant (Thermo Fisher Scientific, Rockford, IL.). Imaging was performed with a Leica DM2000 LED microscope and images were taken with a Leica DFC7000 T camera, at 200 or 400x magnification.

For quantification, 5 fields from the hippocampal formation from each case (n=5 for non-AD and, p-preAD, n=7 for sympAD, n=4 for FTLD-TDP cases) were taken at 200x using a Leica DFC7000 T camera. pTDP43-positive neurons were counted, and from these, p- τ -positive cells were also counted. A ratio (p- τ and pTDP43-positive neurons/total pTDP43-positive neurons) was performed and the results are shown as an average of the 5 fields, in percentages.

TDP-43 pathology analysis

The following neuropathological lesions positive for pTDP-43 antibodies (Suppl. Table 2) were considered: neuronal cytoplasmic inclusions (NCIs), dystrophic neurites (DNs), neuronal intranuclear

inclusions (NII) or neurofibrillary tangle-like material. For the assessment of LATE-NC stage, i.e. cases with TDP-43 pathology in predominantly limbic regions (amygdala and hippocampal formation) without exclusive FTD-related symptoms [11]. The amygdala, hippocampal formation and frontal cortex were used to stage for LATE-NC as proposed: Stage 0 = no LATE-NC; stage 1 = TDP-43 pathology restricted to the amygdala; stage 2 = TDP-43 pathology in the amygdala and hippocampal formation; stage 3 = TDP-43 pathology extending to the frontal cortex, in addition to stage 2 regions. LATE-NC staging was based on sections stained with a polyclonal antibody against pTDP-43 (pS409/pS410, Cosmobio). These criteria were not applied to the FTLD-TDP cases (positive controls). For the assessment of pTDP-43 and p- τ NFT severity analysis in the hippocampus (Fig. 1b), a semi-quantitative assessment was used, as follows: mild (1), moderate (2), or severe (3). Inclusions were considered “mild” when only a few lesions were present in 20 $\times$ microscopic fields, “moderate” when multiple lesions were present in every 20 $\times$ microscopic field, and “severe” when abundant lesions were present in every 20 $\times$ microscopic field. It is essential to note that the semiquantitative scores for pTDP-43 and p- τ pathology cannot be directly compared as the maximum pathology observed for each lesion was considered as maximum score (3), respectively.

A β and τ pathology assessment

To determine the anatomical distribution of A β plaques, phases of A β deposition in the medial temporal lobe (A β MTL phases) were assessed as follows: A β plaques in the temporal neocortex (layers III, V and VI) characterize A β MTL phase 1. In A β MTL phase 2, the plaque deposition spreads into the layers pre- β – pri- γ of the entorhinal cortex, CA1, and the subiculum. A β MTL phase 3 is characterized by A β deposition in all six layers of the temporal neocortex including subpial band-like A β accumulation. In addition, A β plaques also occur in the outer molecular layer of the dentate gyrus and the parvopyramidal cell layer of the presubicular region. Finally, in A β MTL phase 4 there is fully developed β -amyloidosis in the MTL with additional A β plaques in the CA4 region of the hippocampus and in the pre- α layer of the entorhinal cortex [13].

NFT distribution was assessed using the Braak NFT-staging method: Stage I is characterized by p- τ -positive neurons and threads, limited to the transentorhinal region, stage II by p- τ pathology in the entorhinal region, extending to CA1 and CA2, stage III by affection of the neocortex of the fusiform and lingual gyri, stage IV by progression into neocortex-associated areas and the dentate gyrus, stage V by the involvement of frontal and occipital cortex, reaching the peristriate region (layer V also begins to be affected) and finally, stage VI is identified by p- τ pathology in secondary and primary neocortical areas and extends into the striate area of the occipital lobe. CAA was diagnosed whenever A β deposits were found in the wall of cerebral and leptomeningeal blood vessels [4]. NFT pathology in the absence of A β plaque pathology was classified as definite primary age-related tauopathy (PART) [3].

Proximity ligation assays (PLA)

Paraffin-embedded hippocampal slides from 3 non-AD, 3 sympAD and 3 FTL-D-TDP cases underwent low pH pre-treatment, de-paraffinization and the protocol according to the manufacturer's instructions was followed (Duolink® PLA Fluorescence Protocol, Merck, St. Louis, Missouri, US). Briefly, the slides were incubated with Duolink® Blocking solution at 37°C for 1h. The slides were then incubated with an antibody cocktail overnight: rabbit pTDP-43 (Cosmobio, Japan) + mouse p- τ (clone AT8). The tissue was incubated with Duolink® Probes (PLUS and MINUS) for 1h at 37°C. Ligation was made with ligase enzyme diluted in Duolink® Ligation buffer for 30 minutes at 37°C, followed by amplification with polymerase enzyme diluted in Duolink® Amplification buffer, for 100 minutes at 37°C. The slides were finally washed, mounted with ProLong mounting medium with DAPI (ThermoFisher Scientific, Rockford, IL) and visualized using a Leica DM2000 LED microscope and images were taken with a Leica DFC7000 T camera, at 200x magnification.

TDP-43 extraction and biochemical fractionation

The human autopsy brains from five non-AD, 5 pre-clinical AD, 8 sympAD and 5 FTL-D-TDP cases were used in this study. The right hemispheres were cut in approximately 1cm slabs and frozen at -80°C. 100mg of the entorhinal cortex punctures were weighed and TDP-43 was extracted in a previously described manner [12]. Briefly, grey matter was solubilized in a high-salt buffer (10mM Tris-HCL, 0.5M NaCl, 10% sucrose, 1mM DTT, 1% Triton-X and a cocktail of protease/phosphatase inhibitors (Halt, Thermo fisher Scientific, Rockford, IL) followed by ultra-centrifugation. Myelin was removed in 20% sucrose, high-salt buffer, after ultra-centrifugation. The pellet was further resuspended in 2% sarkosyl in high-salt buffer, containing Pierce Universal Nuclease (0.1ug/mL, Thermo fisher Scientific, Rockford, IL) for 45 minutes and ultra-centrifuged. The resulting pellet was washed in PBS twice. The final pellet was resuspended in 300uL of PBS, sonicated and kept as a sarkosyl-insoluble fraction. The total protein concentration of this fraction was measured with BCA assay (Thermo fisher Scientific, Rockford, IL). This sarkosyl-insoluble fraction was selected due to the presence of high amounts of pathological, insoluble aggregates, such as p- τ NFTs and TDP-43 material [1, 5, 8, 12]. Moreover, the use of urea or formic acid as detergent might dissolve proteins that are bound to one another due to conformation-dependent binding sites. Therefore, we decided to use the less harsh sarkosyl-based homogenization protocol.

Western blotting

15ug of total protein of sarkosyl-insoluble extracts were loaded into NuPAGE 4-12% gels (Thermo fisher Scientific, Rockford, IL), after protein denaturation (NuPAGE LDS sample buffer and NuPAGE sample reducing agent (Thermo fisher Scientific, Rockford, IL)). Electrophoresis was performed using MOPS-SDS running buffer and proteins were transferred into 0.2 um nitrocellulose membranes (GE Lifesciences). The membranes were then blocked in TBS-T, containing 5% BSA and incubated

overnight at 4 °C with rabbit polyclonal anti-TDP43 antibody or mouse monoclonal anti-p- τ (PHF1, pS396/pS404). Membranes were washed three times with PBS-T and incubated with respective secondary antibodies: HRP goat anti-rabbit or HRP goat anti-mouse. Immunoblots were washed with PBS-T three times and detected using the SuperSignalTM WestDura substrate (Thermo fisher Scientific, Rockford IL) and an Amersham Imager 600 (GE Life Sciences).

Immunoprecipitation assays

350 μ g of total protein of sarkosyl-insoluble extracts were immunoprecipitated with 2 μ g of mouse monoclonal anti-p- τ (pS202/pThr205, clone AT8, Thermo Scientific, Rockford, IL) using magnetic DynabeadsTM M-280 sheep anti-mouse (Thermo fisher Scientific, Rockford IL). Briefly, the beads were washed three times in PBS containing 0.1% BSA and 0.2 μ M EDTA, and incubated with the appropriate antibody overnight at 4° C. For antigen-binding, sark-insoluble brain extracts from entorhinal cortex were added and incubated for 2 hours at 4°C, with constant rotation. After washing, the immunoprecipitates were eluted with LDS sample buffer 2x (NuPAGE LDS sample buffer) at 95°C. To validate the immunoprecipitation, the immunoprecipitates were immunoblotted (as previously described) with monoclonal mouse anti-human τ (H7T). To address interactions between pTDP-43 and p- τ , the precipitates were immunoblotted with rabbit polyclonal anti-TDP43 (1:1000, Thermo fisher Scientific, Rockford, IL) or with mouse monoclonal anti-p- τ (PHF1, pS396/pS404) (1:2000, Dr. Peter Davies lab), respectively. A negative control per disease group was included consisting on 350 μ g of total protein of sarkosyl-insoluble extracts, magnetic beads but no primary antibody.

Semiquantitative analysis of western blots

Immunoblot bands (43kDa and 45kDa TDP-43) were quantified using ImageJTM software, by measuring the area under the curve for each individual band. Reported values correspond to the respective ratios of the respective 43 and 45 kDa TDP-43 band densities (Fig. 1j, k, l) permitting comparison among the cases analyzed.

Statistical Analysis

Statistical analysis was performed with SPSS 27 software (IBM). Non-parametric, Kruskal-Wallis tests were performed to compare the severity of p- τ NFTs and pTDP-43 inclusions, the amount of co-localization between p- τ and pTDP-43, and to compare the ratios of TDP-43 immunoblot bands. Bonferroni corrections for multiple testing were performed in all instances. *p<0.05, **p<0.01.

Supplementary Table 1- Human cases used in this study. Abbreviations; NA: not applicable; m: male; f: female; PART: primary age-related tauopathy; CAA: cerebral amyloid angiopathy; AGD: argyrophilic grain disease; LBD: Lewy body disease; immunohistochemistry; IF: immunofluorescence; WB: western blot; IP: immunoprecipitation.

Case no.	Age	Sex	Post-mortem diagnosis	Clinical diagnosis	CDR score [9, 10]	Braak NFT stage [2]	A β MTL phase [13]	LATE-NC stage [11]	Cased used for:
1	61	m	Non-AD	Non-demented	0	0	0	1	IHC
2	69	f	Non-AD	Non-demented	0	0	0	0	IHC
3	68	m	Non-AD (+PART)	Non-demented	0	I	0	0	IHC
4	74	m	Non-AD (+infarcts, microinfarcts)	Non-demented	0	0	0	0	IHC, PLA,IP,WB
5	36	m	Non-AD	Non-demented	0	0	0	0	IHC
6	69	f	Non-AD (+PART)	Non-demented	0	I	0	2	IHC
7	59	f	Non-AD (+PART, CAA)	Non-demented	0	I	0	0	IHC,PLA,IP,WB
8	54	m	Non-AD (+PART)	Non-demented	0	I	0	0	IHC,IP,PLA,WB
9	56	m	Non-AD (+PART)	Non-demented	0	I	0	0	IHC,IP,WB
10	76	m	Non-AD (+PART)	Non-demented	0	I	0	0	IHC,WB
11	67	f	Non-AD (+PART)	Non-demented	0	I	0	0	IHC
12	78	m	Non-AD (+PART)	Non-demented	0	II	0	2	IHC
13	79	f	Non-AD (+infarcts)	Non-demented	0	II	0	2	IHC,IF,IP,WB
14	78	f	p-preAD (+CBD)	Non-demented	0	I	1	2	IHC
15	70	m	p-preAD	Non-demented	0	I	1	2	IHC
16	73	f	p-preAD	Non-demented	0.5	I	1	1	IHC
17	83	f	p-preAD (AGD)	Non-demented	0	III	1	2	IHC,IF
18	72	m	p-preAD	Non-demented	0	III	2	1	IHC,IF
19	67	m	p-preAD	Non-demented	0	II	3	1	IHC
20	85	m	p-preAD (+AGD)	Non-demented	0	III	2	2	IHC,IF,IP,WB
21	83	f	p-preAD	Non-demented	0	IV	3	2	IHC,IF,IP,WB
22	77	m	p-preAD	Non-demented	0	II	1	2	IHC,IP,WB
23	78	f	p-preAD (+infarcts, CAA, LBD)	Non-demented	0	III	4	2	IHC,IP,WB
24	78	m	AD (+CAA)	AD	3	IV	4	2	IHC

25	72	f	AD	AD	1	VI	4	2	IHC
26	82	m	AD	AD	2	III	4	2	IHC
27	81	f	AD (+CAA)	AD	3	V	4	2	IHC
28	83	m	AD	AD	3	IV	4	2	IHC
29	87	m	AD	AD	2	VI	4	2	IHC
30	71	m	AD	AD	3	V	4	2	IHC,IF,PLA ,IP,WB
31	57	m	AD	AD	3	VI	4	2	IHC,IF,PLA ,IP,WB
32	55	f	AD	AD	3	VI	4	2	IHC, IF,WB
33	86	m	AD (+infarcts, microinfarcts)	AD	2	V	4	2	IHC,IF,IP, WB
34	76	m	AD	AD	3	V	4	3	IHC,IP,WB
35	77	f	AD (+LBD)	AD	2	V	4	3	IHC,IF,IP, WB
36	74	m	AD	AD	2	VI	4	3	IHC,IF,WB
37	71	f	AD	AD	3	VI	4	3	IHC,WB
38	76	m	AD	AD	3	V	4	2	IHC,IF,PLA ,IP
39	74	f	AD	AD	na	VI	4	2	IHC
40	87	m	AD	AD	na	IV	4	3	IHC
41	64	m	AD	AD	1	VI	4	2	IHC
42	86	f	A (+AGD)	AD	2	IV	4	2	IHC
43	78	f	AD	AD	1	V	4	2	IHC
44	89	f	AD (+CAA)	AD	2	IV	4	2	IHC
45	89	f	AD (+CAA)	AD	3	V	4	3	IHC
46	83	m	AD (+CAA)	AD	2	V	4	2	IHC
47	60	f	AD	AD	3	V	4	2	IHC
48	76	m	AD	AD	na	VI	4	2	IHC
49	79	f	FTLD-TDP type A	bvFTD	3	I	0	NA	IHC,IF,PLA ,IP,WB
50	62	f	FTLD-TDP type C	svPPA	3	0	0	NA	IHC,IF,IP, WB
51	69	f	FTLD-TDP type C	svPPA	2	I	0	NA	IHC,IF,PLA ,IP,WB
52	64	f	FTLD-TDP type A	bvFTD	2	I	0	NA	IHC,IF,PLA ,IP,WB
53	58	m	FTLD-TDP type B	bvFTD	3	I	0	NA	IHC,IP,WB
54	81	f	FTLD-TDP type A (+AGD)	svPPA	2	II	2	NA	IHC,IF
55	38	m	FTLD-TDP type A	bvFTD	2	I	0	NA	IHC
56	63	m	FTLD-TDP type A	PSP	3	II	3	NA	IHC
57	58	f	FTLD-TDP type B (+AGD)	bvFTD	3	I	0	NA	IHC

Supplementary Table 2 – Primary antibodies used in this study

Antibody	Epitope	Dilution (application)
pTDP-43, polyclonal rabbit (CosmoBio)	pS409/pS410	1:5000 (IHC) 1:1000 (IF)
C-t TDP-43, polyclonal rabbit (Proteintech)	260-414aa.	1:1000 (IHC) 1:500 (IF)
C-t TDP-43 (Invitrogen)	350-414aa.	1:1000 (WB)
N-t TDP, polyclonal rabbit (AVIVA systems)	1-50aa.	1:500 (IHC) 1:400 (IF)
p- τ , clone PHF-1, mouse (Gift from Dr. Peter Davies)	pS396/pS404	1:2000 (WB) 1:500 (IHC)
p- τ , clone AT8, mouse (ThermoFisher Scientific)	pS202/pThr205	1:150 (IF)
Abbreviations; IHC: immunohistochemistry; IF: immunofluorescence; WB: western blot		

Supplementary Table 3 – P-values for non-parametric, Kruskal-Wallis tests with Bonferroni corrections to compare the severity of immunohistochemically observed hippocampal pTDP-43 pathology (pS409/pS410) among the different disease groups. Adjusted p values for multiple testing are in parenthesis. Non-AD (n=12), p-preAD (n=13), sympAD (n=25) and FTLD-TDP (n=9).

	Non-AD	p-preAD	AD	FTLD-TDP
Non-AD	-	0.029 (0.177)	0.006 (0.033)	0.0001 (0.003)
p-preAD	-	-	0.954 (1)	0.348 (1)
AD	-	-	-	0.284 (1)

Supplementary Table 4 – P-values for non-parametric, Kruskal-Wallis tests with Bonferroni corrections to compare the severity of immunohistochemically observed hippocampal p- τ NFT pathology (clone AT8, pS202/pThr205) among the different disease groups. Adjusted p values for multiple testing are in parenthesis. Non-AD (n=12), p-preAD (n=13), sympAD (n=25) and FTLD-TDP (n=9).

	Non-AD	p-preAD	AD	FTLD-TDP
Non-AD	-	0.041 (0.244)	0.0001 (0.001)	0.386 (1)
p-preAD	-	-	0.013 (0.075)	0.326 (1)
AD	-	-	-	0.001 (0.009)

Supplementary Table 5 – P-values for non-parametric, Kruskal-Wallis test with Bonferroni correction to compare the biochemically assessed ratios of 43kDa/45kDa TDP-43 among the different disease groups. Adjusted p values for multiple testing are in parenthesis. Non-AD (n=4), p-preAD (n=4), sympAD (n=5) and FTLD-TDP(n=3).

	Non-AD	p-preAD	AD	FTLD-TDP
Non-AD	-	0.207 (1)	0.005 (0.023)	0.766 (1)
p-preAD	-	-	0.117 (0.705)	0.142 (0.855)
AD	-	-	-	0.003 (0.018)

Supplementary Table 6 – p-values for non-parametric, Kruskal-Wallis test with Bonferroni correction to compare the biochemically assessed ratios of IP 43kDa/input 43kDa TDP-43 among the different disease groups. Non-AD (n=3), p-preAD (n=3), sympAD(n=3) and FTLD-TDP(n=3).

	Non-AD	p-preAD	AD	FTLD-TDP
Non-AD	-	0.174 (1)	0.017 (0.105)	0.374 (1)
p-preAD	-	-	0.308 (1)	0.089 (0.537)
AD	-	-	-	0.007 (0.039)

Supplementary Figure 1 – C- and N-t TDP-43 are present in the nucleus in neurons with τ pathology (arrows). Some NFT-positive neurons display nuclear clearance of TDP-43 (arrowheads). Double-labelling immunohistochemistry of a (a,d) non-AD, (b,e) p-preAD and (c,f) AD case with (a-c) C- or (d-f) N-t TDP-43 antibodies (brown) and p- τ (pS396/pS404 - red). Non-AD (n=2, case 8 is shown), p-preAD (n=2, case 17 is shown), sympAD (n=2, case 34 is shown). Scale bars=50 μ m.

Supplementary Figure 2 – Double-labelling with Gallyas silver staining (black) and pTDP-43 (pS409/pS410) (red) in the CA1 of a sympAD case shows that most Gallyas-positive neurons also co-localize with pTDP-43 (arrows). A few neurons exclusively positive for pTDP-43 (arrowheads) or Gallyas (asterisk) are also observed. AD (n=2, case 34 shown). Scale bars = 50 μ m.

Supplementary Figure 3 - Quantification of co-localization of p- τ and pTDP-43 in the CA1-subfield of the hippocampus relative to the amount of total τ NFTs or pretangles. P-preAD (n=5), AD (n=7) and FTLD-TDP (n=4) cases were used. Non-parametric, Kruskal-Wallis test with Bonferroni correction for multiple testing was used. ** $p < 0.01$.

Supplementary Figure 4 – Full immunoblots used in this study. **(a)** C-t TDP-43 (350-414aa, Invitrogen) immunoblot of sarkosyl-insoluble samples input, including non-AD (n=6), p-preAD (n=4), sympAD (n=8) and FTLD-TDP (n=5) cases. Cases displayed in Fig. 1 f are displayed in a square. **(b)**

p- τ (pS396/pS404) immunoblot of sarkosyl-insoluble samples input, including non-AD (n=5), p-preAD (n=5), sympAD (n=8) and FTLT-TDP (n=5) cases. Cases displayed in Fig. 1 g are displayed in a square. A shorter exposure time regarding this immunoblot is displayed in (c). (d) C-t TDP-43 (350-414aa, Invitrogen) immunoblot of immunoprecipitated (IP), sarkosyl-insoluble samples using an anti-p- τ antibody for immunoprecipitation (AT8, pS202/pThr205, ThermoFisher) including non-AD (n=5), p-preAD (n=3), sympAD (n=6) and FTLT-TDP (n=5) cases. IP negative controls (beads+sample) are indicated with (-). Case used as negative controls for each group in the first immunoblot: p-preAD (case 21), sympAD (case 31), FTLT-TDP (case 49). Cases used as negative controls for each group in the second immunoblot: p-preAD (case 23), sympAD (case 34), FTLT-TDP (case 52). Cases displayed in Fig. 1h are displayed in a square. Unlabeled cases were cases with a distinct FTLT-TDP-like TDP-43 pathology and distinct symptomatology rather than typical AD, therefore were not considered in this study. The other cases used are displayed by the respective case numbers.

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