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Original Article

Caspase-cleaved TAR DNA-binding protein-43 in Pick's disease

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Abstract: The hyperphosphorylation and proteolytic modification of the TAR DNA binding protein-43 (TDP-43) is a key finding in a number of neurodegenerative diseases including frontotemporal dementia with ubiquitin-positive inclusions (FTLD-U), amyotrophic lateral sclerosis (ALS), and most recently Alzheimer's disease (AD). To examine whether proteolytic modifications of TDP-43 is a relevant finding in Pick's disease, we utilized a novel site-directed caspase-cleavage antibody based upon a known caspase-3 cleavage consensus site within TDP-43 at position 219. Application of this antibody, termed TDP caspase-cleavage product (TDPccp) to postmortem Pick's disease brain sections revealed the presence of caspase-cleaved TDP-43 in Pick and Hirano bodies predominantly within region CA1 of the hippocampus. Co-localization of TDPccp with PHF-1, a general marker for Pick bodies, as well as with an antibody to caspase-cleaved tau (TauC3) was evident within the hippocampus. A semi-quantitative analysis indicated that approximately 21% and 79% of the Pick bodies identified in area CA1 contained caspase-cleaved TDP-43 or caspase-cleaved tau, respectively. Of interest was the lack of co-localization of TDPccp with PHF-1 in Pick bodies within the dentate gyrus. Collectively, these data have identified modified TDP-43 as a component of Pick and Hirano bodies that is restricted to area CA1 in Pick's disease. The relative paucity of caspase-cleaved TDP-43 found within Pick bodies in comparison to caspase-cleaved tau suggests that TDP-43 and its modification by caspases is most likely not a contributing factor leading to Pick body formation.

Key Words: Pick's disease, Pick bodies, caspases, TDP-43, Hirano bodies, tau

Introduction

TAR DNA binding protein-43 (TDP-43) inclusions have recently been identified as a major feature of several neurodegenerative disorders including frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) and amyotrophic lateral sclerosis (ALS), [1]. A conspicuous finding in these studies was the presence of 25 and 35 kDa truncated fragments of TDP-43 in brain extracts from affected individuals that were not present in control subjects [1,2]. A similar 25 kDa, truncated fragment of TDP-43 has also been identified in the Alzheimer's disease (AD) brain [3]. Thus, post-translational proteolytic processing of TDP-43 may be a key step in protein misfolding and aggregation of TDP-43 leading to a toxic gain of function [4]. Recently, it has been determined that caspase-3 may be the protease involved in processing TDP-43 mediated through an interaction with progranulin [2]. These authors demonstrated that altered cleavage of TDP-43 in cell culture models led to the production of 25 kDa fragments of TDP-43 that were similar in appearance to those found in FTLD-U and ALS [2]. Therefore, abnormal turnover of TDP-43 may underlie the neurodegeneration observed in these disorders.

We recently developed a site-directed caspase-cleavage antibody to TDP-43, which we termed TDPccp, and application of this antibody to postmortem AD brain sections labeled tangles, plaques, reactive astrocytes and Hirano bodies [5]. The purpose of the present study was to determine a possible role for caspase-cleaved TDP-43 in an additional tauopathy, namely Pick's disease. Filamentous neuronal and glial hyperphosphorylated tau are the defining neuropathological characteristics associated

Table 1. Case Demographics

Case	Group	Sex	Age	PMI (hrs)	BraakNFT	MMSE
1	Pick	M	66	5.0	3	0
2	Pick	M	72	2.5	0	N/A
3	Pick	F	76	3.3	0	5
4	Pick	F	68	3.2	0	N/A
5	Pick	F	70	3.5	N/A	N/A

Note. PMI, postmortem interval; MMSE, mini mental state examination; N/A, not available

with Pick's disease [6]. Pick's disease is associated with severe neuronal and glial loss leading to frontotemporal lobe atrophy [6]. Pathologically, a key feature of Pick's disease is the presence of Pick bodies representing intracellular inclusions containing aggregates of hyperphosphorylated tau [7]. Previous studies have identified the presence of TDP-43 inclusions in Pick's disease [8, 9].

In the present study, application of our site-directed caspase-cleavage antibody to TDP-43 revealed labeling that was more or less restricted to CA1 region of the hippocampus in Pick's disease. Intense labeling within Hirano bodies and reactive astrocytes was similar to what we observed previously in AD [5]. Roughly, 21% of the total number of Pick bodies identified within area CA1 contained caspase-cleaved TDP-43. These results suggest the presence of modified TDP-43 is a consistent finding in tauopathies including AD and Pick's disease, however, given the relative paucity of caspase-cleaved TDP-43 labeling within Pick bodies, inclusions of TDP-43 may not be a causative factor in Pick body formation.

Materials and Methods

Materials

The mouse TauC3 antibody (caspase-cleaved tau antibody) was purchased from Invitrogen/ Chemicon (Carlsbad, CA). The caspase-cleavage product antibody to TDP-43 (TDPccp) was an in house antibody synthesized based upon a putative caspase cleavage consensus site ($DVMD^{219}$) within TDP-43. This antibody has previously been shown to be a specific marker for caspase-cleaved TDP-43 [5]. PHF-1 was a generous gift from Dr. Peter Davies (Albert Einstein College of Medicine, Bronx, NY).

Human subjects

Autopsy brain tissue from five neuropathologically confirmed Pick's cases was studied. Case demographics are presented in **Table 1**. Human brain tissues used in this study was provided by the Institute for Brain Aging and Dementia Tissue Repositories at the University of California, Irvine.

Immunohistochemistry and immunofluorescence microscopy

Free-floating 40 μm-thick serial sections were used for immunohistochemical and immunofluorescence studies as previously described [10]. Antibody dilutions were the following: TDPccp (1:100), PHF-1 (1:500), and mAb TauC3 (1:100). Antigen visualization was determined using ABC complex (ABC Elite immunoperoxidase kit, Vector labs), followed by brown/red DAB substrate (Vector Labs) for single labeling. Visualization of bright-field double label immunohistochemistry was accomplished using brown DAB for one label and blue SG substrate (Vector Labs) for the second label. For immunofluorescence co-localization studies, antigen visualization was accomplished using an Alexa fluor 488-labeled tyramide (green, Ex/Em = 495/519) for one label and streptavidin Alexa Fluor 555 (red, Ex/Em = 555/565) for the second label, both from Invitrogen (Carlsbad, CA).

Quantification and statistical analysis

A semi-quantitative analysis was performed by first taking 20X immunofluorescence, overlapping images from three different fields in area CA1 of the hippocampus in three separate Pick's cases. Photographs were then analyzed by counting the number of inclusion bodies per field for each case. Data were than averaged ±S.E.M. Additionally, PHF-1 labeling

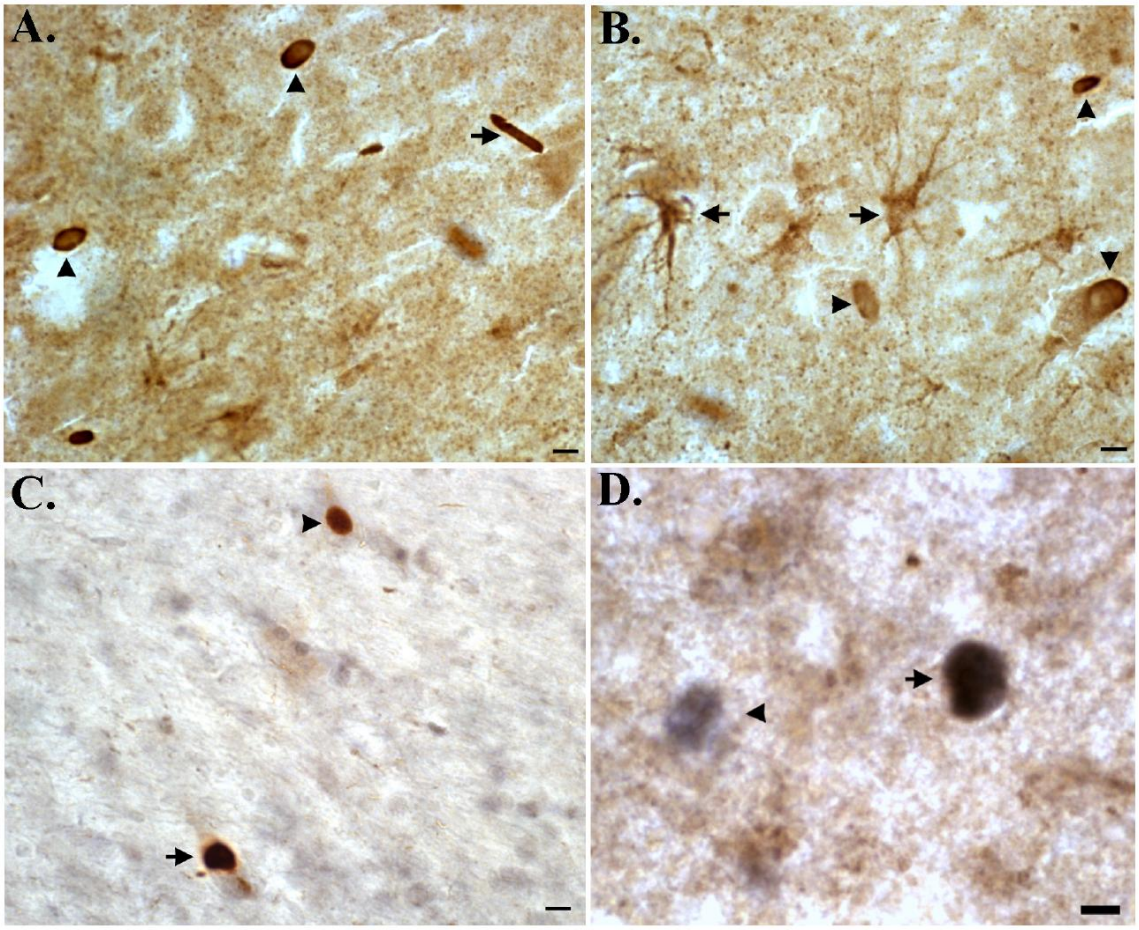

Figure 1. Caspase-cleaved TDP-43 in Pick's disease. A and B: Representative single labeling from a Pick's case utilizing the TDPccp antibody illustrating staining in the CA1 region of the hippocampus within Pick bodies (arrowheads, A and B), Hirano bodies (arrow, A) and astrocytes (arrows, B). C and D: Representative double-labeling from a Pick's case employing TDPccp antibody and PHF-1 within the substantia nigra (C) and amygdala (D). A sparse number of Pick bodies was detected in both brain regions, and in some cases co-localized with PHF-1 (arrows, C and D). All scale bars represent 10 μm.

was utilized as a general marker for Pick bodies and compared with the number of PHF-1-labeled Pick bodies that co-localized with either TauC3 or TDPccp. Data are representative of the average number of TDPccp or TauC3 Pick bodies co-localized with PHF-1 in each 20X field (3 fields total for 3 different cases). In a parallel experiment, we also determined the percent of Pick bodies that were identified as having both TDPccp and TauC3 present. For determining statistical differences, the degree of colocalization of PHF-TDPccp and PHF-TauC3 double-labeled Pick Bodies were analyzed by Kruskal-Wallis nonparametric alternative test for a 1-way ANOVA with statistical significance established at a p-value < 0.05. All calculations were performed using a SAS program version 9.1.3 Service Pack 3 for Windows XP-Pro.

Results and Discussion

Recent advances have suggested that in TDP-43 proteinopathies including FTLD-U and ALS, a key event that may promote disease progression is the redistribution of TDP-43 from the nucleus to the cytoplasm [11]. In addition, brain samples from both FTLD-U and ALS are enriched in smaller (~25 kDa) phosphorylated fragments of TDP-43, suggesting that posttranslational modification of TDP-43 may signal a shift to a toxic gain of

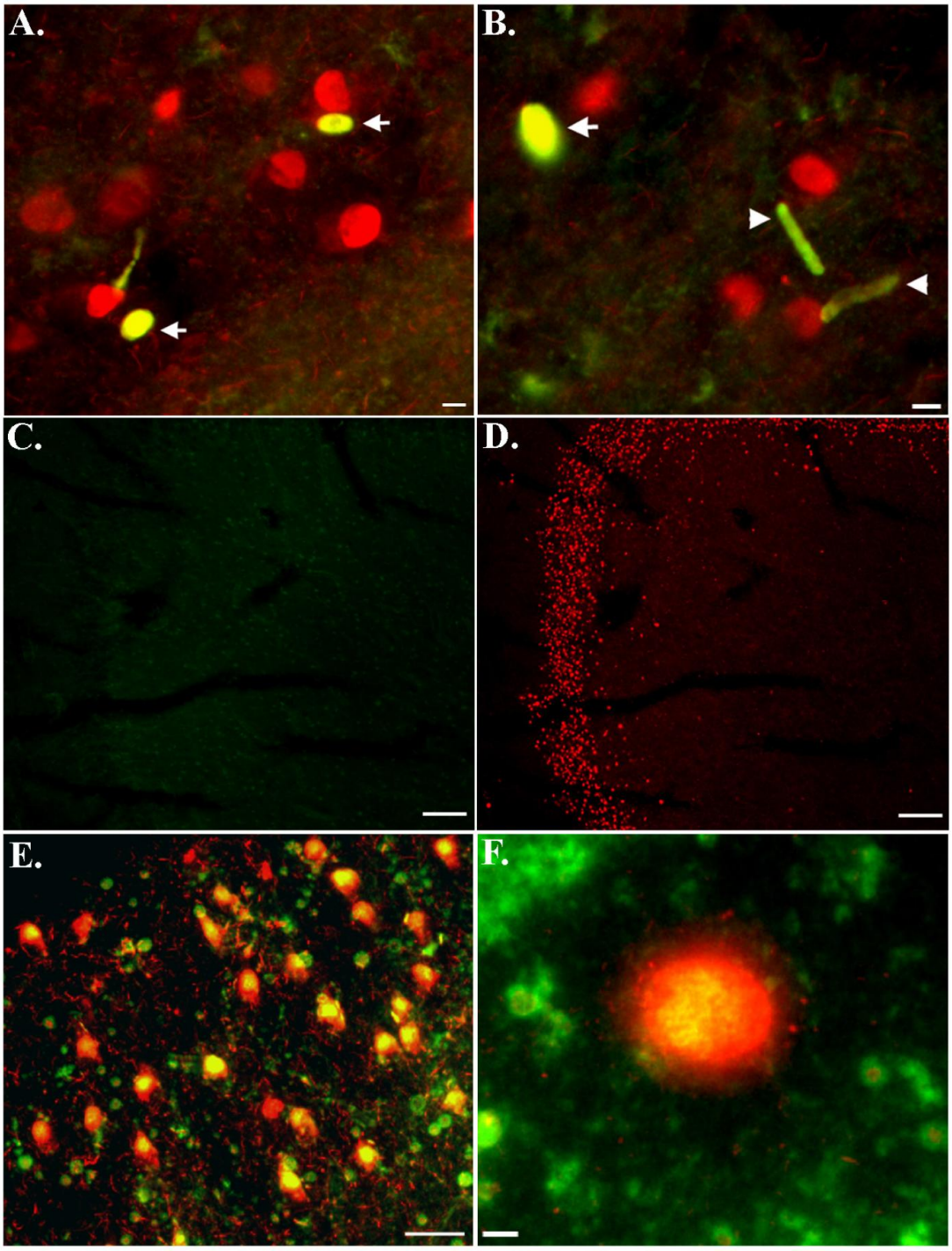

Figure 2. Caspase-cleaved TDP-43 labeling within the hippocampus of Pick's disease is uniform and regionally defined. A-D: Representative immunofluorescence double-labeling utilizing the TDPccp antibody (green) and PHF-1 (red) revealed the presence of caspase-cleaved TDP-43 within Pick (arrows, A) and Hirano bodies (arrowhead, B) predominantly within area CA1 (A and B), but not within granule cells of the dentate gyrus (C). PHF-1 labeling was evident throughout all areas of the hippocampus and widespread within the dentate gyrus (D). A small percentage of Pick bodies were co-localized with both antibodies (arrows, A and B). E and F: Representative immunofluorescence double-labeling within the CA1 region of the hippocampus in Pick's disease utilizing TauC3 (green) and PHF-1 (red). Unlike TDPccp, which appeared to reveal a more uniform staining pattern, TauC3 staining was localized centrally within Pick bodies and displayed a more aggregated appearance. Scale bars are 10 μm in A, B, and F, 100 μm in C and D, and 20 μm in E.

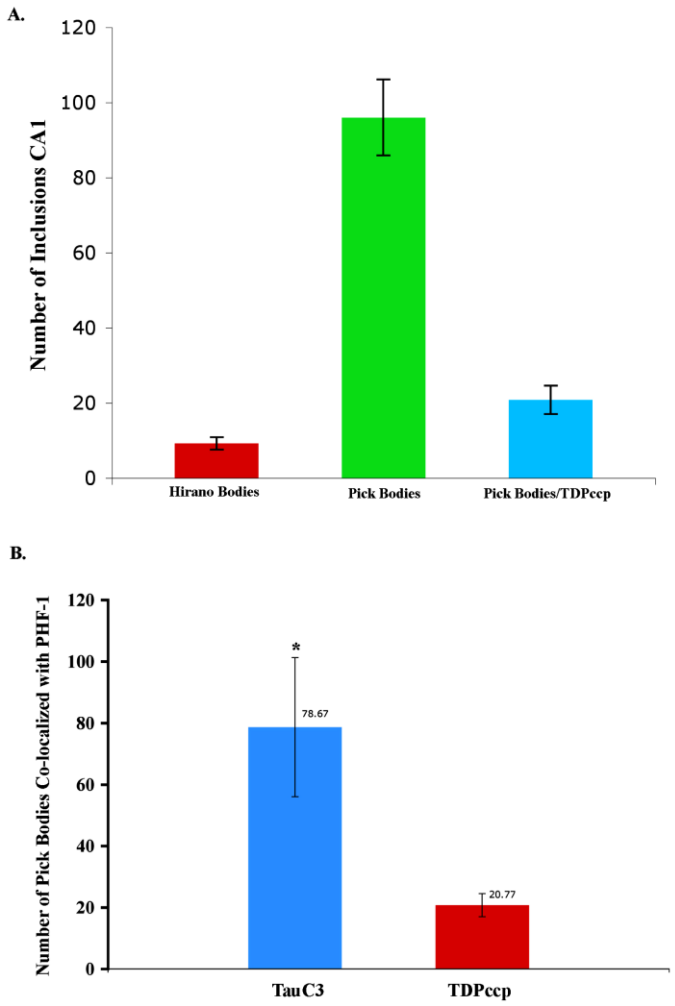

Figure 3. Quantification of Pick bodies double-labeled by TDPccp, TauC3, and PHF-1. In both A and B, PHF-1 labeling was utilized as a marker for Pick bodies. A: Data show the average number of Hirano bodies labeled with TDPccp, Pick bodies labeled with PHF-1, and Pick bodies with both PHF-1 and TDPccp, identified in a 20X field of area CA1 (n=3 fields for 3 different Pick disease cases) ±S.E.M. B: a semi-quantitative analysis was performed to estimate the total number of PHF-1-labeled Pick bodies that co-localized with either TDPccp or TauC3 within the CA1 region of the hippocampus (percent given above bars). Results indicated the total number of double-labeled Pick bodies with caspase-cleaved tau plus phosphorylation was significantly greater compared with caspase-cleaved TDP-43 plus phosphorylation. Data represent the average (±S.E.M.) of three different fields from three representative Pick cases ($\chi 2$ = 3.857, p-value* < 0.05).

function [12]. One candidate protease that may be involved in the proteolytic processing of TDP-43 is caspase-3. Zhang et al. have shown that TDP-43 is a substrate for caspase-3 leading to the generation of 25 and 35 kDa species [2]. Further, utilizing an in vitro model, the authors demonstrated a caspase-dependent cleavage and redistribution of TDP-43 from its nuclear localization to the cytoplasm [2]. Our lab has also supported the hypothesis that caspases may play a major role in the processing of TDP-43 in the AD brain: utilizing a site-directed caspase-cleavage antibody to a known consensus cleavage site within TDP-43 (DVMD^{219}), we showed strong immunoreactivity within plaque regions, tangles, reactive astrocytes and Hirano bodies of the hippocampus [5]. To confirm and extend these findings, we examined whether caspase-cleaved TDP-43 is a finding in the Pick's disease. Like AD, Pick's disease is classified as a tauopathy characterized pathologically by the presence of round Pick body structures containing predominantly hyperphosphorylated, aggregated tau [13].

Five confirmed cases of Pick's disease (Table I) were examined by immunohistochemistry utilizing our caspase-cleaved antibody to TDP-43, termed TDPccp. As an initial approach, immunoreactivity to TDPccp was assessed in three different brain regions including the hippocampus, amygdala, and substantia nigra. As depicted in **Figure 1**, we detected labeling of TDPccp within numerous Pick bodies in the hippocampus (**Figure 1A and B**) as well as within the substantia nigra and amygdala although in the latter case in much fewer numbers (**Figure 1C and D**). The localization of TDPccp within Pick bodies in the substantia nigra and amygdala was confirmed following double-labeling with PHF-1, a general marker for Pick bodies (arrows, **Figure 1C and D**). In addition to Pick body labeling, staining of TDPccp in the hippocampus was also observed in Hirano bodies (arrow, **Figure 1A**) and reactive astrocytes (arrows, **Figure 1B**).

The staining of TDPccp within Hirano bodies was similar to what we previously observed in AD, being largely confined to area CA1 of the hippocampus [5]. Hirano bodies are rod-

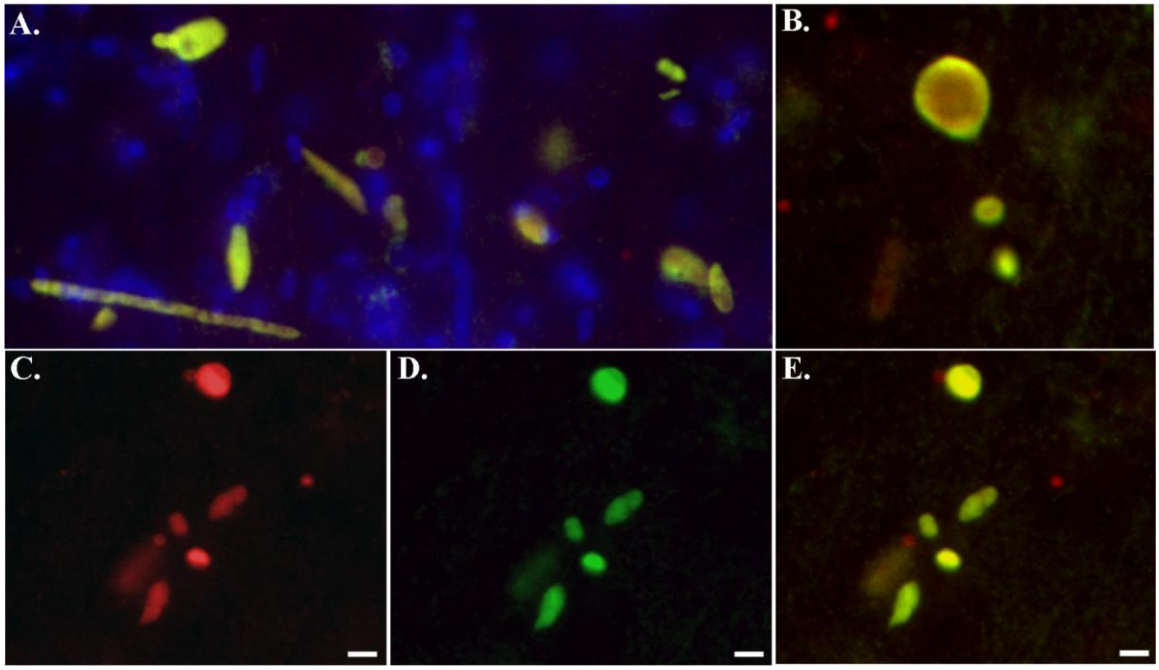

Figure 4. Caspase-cleaved tau and TDP-43 co-localization within Pick bodies. A and B: Representative immunofluorescence double-labeling employing the TDPccp antibody (green) and TauC3 (red) in CA1. Panel A also displays nuclear staining using Hoest (blue). Note the relative uniform distribution of TDPccp while TauC3 appeared to be less uniform and in some cases more centrally distributed within the Pick body (B). Co-localization was also observed occasionally within Hirano bodies (A). C-D: Identical to Panels A and B showing TauC3 labeling (red, C), TDPccp (green, D), and the overlapped image for both markers (yellow, E). Scale bars represent 10 μm.

shaped, cytoplasmic inclusions that are found predominantly within the hippocampus in a variety of neurodegenerative diseases, including AD and Pick's disease [14]. The function of Hirano bodies is unknown as is whether the presence of these structures contributes to observed neurodegeneration in various neurodegenerative disorders. Thus, the finding of caspase-cleaved TDP-43 within Hirano bodies of the AD and Pick's disease brain suggest this might be a common feature of tauopathies.

To examine the relative and regional distribution of TDPccp labeling within the hippocampus, double-label immunofluorescence studies were carried out utilizing PHF-1 as a general marker for Pick bodies. Co-localization of both antibodies was evident within Pick bodies in area CA1 and the staining pattern revealed a homogenous, uniform labeling for TDPccp (arrows, **Figure 2A**). Interesting, although double-labeling within Pick bodies was observed, Hirano bodies were labeled only with TDPccp (arrowhead, Fig. 2B). Numerous Pick bodies identified by PHF-1 staining were evident within the granule cell layer of the dentate gyrus (**Figure 2D**). However, there was a complete lack of labeling with TDPccp in the same area (**Figure 2C**), indicating that caspase-cleaved TDP-43 is regionally restricted to Pick and Hirano bodies within the CA1 region of the hippocampus. A semi-quantitative analysis indicated that approximately 21% of the total number of Pick bodies identified following labeling with PHF-1 contained caspase-cleaved TDP-43 (**Figure 3**).

To determine whether other known substrates for caspase cleavage are present within Pick bodies, experiments were undertaken using an antibody that specifically detects caspase-cleaved tau (TauC3) [15]. In a previous study, it was shown that a large fraction of Pick bodies (~90%) were double-labeled with both PHF-1 and TauC3 [16]. Confirming these findings, in the present study co-localization of PHF-1 and TauC3 was evident within Pick

bodies in the hippocampus (**Figure 2E and F**). In general, the staining pattern was different than what was observed for TDPccp: TauC3 labeling was centrally located within the Pick body and displayed an aggregated, fibrillar pattern of staining (**Figure 2F**). In addition, a much larger fraction of Pick bodies were double-labeled for PHF-1 and TauC3 (79%) in comparison to TDPccp (21%) (**Figure 3**). Taken together, these results suggest the caspase-cleavage of tau may promote its ability to aggregate, an observation that has been identified as a putative mechanism for tangle formation in the AD brain [15, 17]. Moreover, because a much larger fraction of Pick bodies are TauC3-positive, this supports the idea that the caspase cleavage of tau may be an important event in the formation and evolution of Pick bodies.

In a final set of experiments, we evaluated whether co-localization of TauC3 and TDPccp was evident in Pick bodies of the hippocampus. As shown in Figure 4, double-labeling of these antibodies was evident within both Pick and Hirano bodies. Similar to what was observed previously, TauC3 labeling was non-uniform in appearance, and was centrally located within Pick body structures (Fig. 4A and B). A semi-quantitative analysis revealed that approximately 46% ±13% of identified Pick bodies contained both caspase-cleaved tau and TDP-43, with the remaining 54% of Pick bodies appeared to be single-labeled with only TauC3.

In conclusion, we have demonstrated the presence of caspase-cleaved TDP-43 within Pick and Hirano bodies of the Pick's disease brain. These findings support the conclusion that the presence of TDP-43 pathology is not solely restricted to TDP-43 proteinopathies, but may be more widely distributed in a number of neurodegenerative diseases including tauopathies.

The presence of caspase-cleaved TDP-43 within Hirano bodies is similar to our previous findings in AD, and suggests that this may be a common finding in various tauopathies. Further studies will be necessary to elucidate the relationship between TDP-43 and the formation and evolution of Hirano bodies. Previous studies have demonstrated that Hirano bodies are rich in cytoskeletal proteins including actin and tau [18, 19]. Further, a previous study by Wang et al., indicated the presence of TDP-43 within the somatodendrites of hippocampal neurons where it co-localized with beta-actin mRNA [20]. Based on these findings it is interesting to speculate on a role of TDP-43 in the regulation of the cytoskeleton, a role that may be disrupted following the activation of caspases and cleavage of TDP-43 in various neurodegenerative disorders including AD and Pick's disease.

Another finding of the present study was the presence of TauC3 and TDPccp within Pick bodies in the hippocampus of affected patients. Our data clearly demonstrated a staining pattern for TauC3 that was fibrillar in appearance, and supports previous studies that the caspase cleavage of tau may promote the ability of tau to aggregate into beta-sheet structures [15, 17]. Whether the caspase-cleavage of tau promotes Pick body formation in Pick's disease is unknown, but based upon the large fraction of Pick bodies containing caspase-cleaved tau, it is suggested that this may be an important event underlying the formation of these pathological inclusions. Additional studies are warranted to assess the role of caspase activation and cleavage of tau/TDP-43 in Pick/Hirano body formation.

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