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ORIGINAL PAPER

Astrocytic degeneration in chronic traumatic encephalopathy

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Abstract

Chronic traumatic encephalopathy (CTE) is a neurodegenerative disease associated with repeated head traumas. Using immunohistochemistry for glial fibrillary acidic protein as a marker, plus automated quantitative analysis, we examined the characteristics and extent of astrogliosis present in stage III and IV CTE, along with Alzheimer's disease (AD), and frontotemporal dementia (FTD) cases. Astrogliosis in CTE patients was more diffuse compared to that of AD and FTD patients, which was concentrated in the sulcal depths. Of 14 patients with CTE, 10 exhibited signs of a degenerating astrocyte pathology, characterized by beaded, broken astrocytic processes. This astrocytic degeneration was typically found to be diffuse throughout the white matter, although two cases demonstrated astrocytic degeneration in the gray matter. The degeneration was also observed in 2 of 3 AD and 2 of 3 FTD brains, with overall similar characteristics across diseases. There was minimal to no astrocytic degeneration in six age-matched controls with no neurodegenerative disease. We found that the extent of the white matter astrocytic degeneration was strongly correlated with the level of overall astrogliosis in both the white and gray matter. However, astrocytic degeneration was not correlated with the overall extent of tau pathology. Specifically, there was no correlation between levels of p-tau in the sulcal depths and astrocytic degeneration in the white matter adjacent to the sulcal depths. Thus, astrocytic degeneration and overall astrogliosis appear to represent distinct pathological features of CTE. Further investigation into these astroglial pathologies could provide new insights into underlying disease mechanisms and represent a potential target for in vivo assessment of CTE as well as other neurodegenerative disorders.

Keywords Chronic traumatic encephalopathy · Neurodegeneration · Astrocyte · Glial fibrillary acidic protein

Introduction

Chronic traumatic encephalopathy (CTE) is a neurodegenerative disease associated with patients who have suffered repetitive concussive brain trauma as well as sub-concussive impact, although there have also been reports of CTE in single TBI as well as epilepsy patients [4, 13, 36, 41, 46, 59]. Symptoms associated with CTE include memory loss, impulse control problems, mood disorders, and dementia; subsets also may develop motor neuron disease and Parkinsonism [37, 56]. Current evidence suggests that symptoms can be used to classify subjects with suspected CTE into two classes. The first population typically consists of younger subjects who exhibit behavioral symptoms such as aggression, depression or impulse control deficits, while the second population consists of older subjects who suffer from cognitive deficits resembling those of Alzheimer's disease (AD) [39, 56]. The mechanisms underlying these symptoms are still unclear, and research efforts are hindered by the fact that these symptoms are not specific to the diagnosis of CTE.

Currently, a definitive diagnosis of CTE can only be confirmed post-mortem using immunohistochemical methods to identify key neuropathological features [33]. These include irregularly distributed hyperphosphorylated tau (p-tau) in neurons and astrocytes clustered in the depths of cortical sulci and perivascular regions [20, 33, 36, 45, 46]. Based on the extent and progression of tau pathology, CTE has been proposed to be categorized into four stages (I–IV), although this staging system has not been widely endorsed or independently validated [33–35, 63].

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While there is some correlation between the extent of tau pathology and clinical symptoms, current literature suggests the minimal amount of p-tau found in stage I and II is unlikely to fully account for the extent of cognitive and behavioral impairments found in stages I and II patients. Furthermore, stage of CTE pathology is more strongly correlated with duration of exposure to repeated traumatic brain injuries (rTBI) rather than severity of clinical symptoms [56]. Currently, studies have been inconclusive behind the mechanism causing the spread of tau pathology, with some hypotheses including potential protein misfolding and subsequent prion-like spreading of tau [3, 14]. Similarly, the mechanism underlying the progression of clinical symptoms is also unknown. Studies suggest that axonal shearing, as well as tau hyperphosphorylation and cytoskeletal breakdown may play a role [24, 61], but no studies have proven to be conclusive. A major roadblock to studying the mechanistic pathways in CTE is the lack of validated animal models for CTE. Thus, broader neuropathological investigations are warranted. While tau-positive astrocytes are one of the diagnostic criteria of CTE, there have not been, to our knowledge, extensive studies of the astrocytic pathology in CTE beyond standard p-tau analysis.

When subjected to injury or disease, astrocytes undergo a morphological change, commonly referred to as astrogliosis or astrocytosis. Rather than a uniform reaction in response to insult, astrogliosis has been shown to be a tailored response to specific injuries, with distinct sets of transcriptional changes induced by diverse injuries [23, 26, 67]. These reactive astrocytes have been shown to have a variety of pathophysiological effects. For example, certain reactive astrocytes may inhibit axonal regeneration and produce cytokines that exacerbate injuries [1, 7, 38, 67]. Alternatively, other reactive astrocytes have been reported to enhance recovery from trauma and ischemia [10, 18]. Reactive astrocytes are a key characteristic of AD; astrocytes may help clear and degrade amyloid β [27], and the degree of astrogliosis has been reported to be positively correlated with Braak stages in AD [52, 53]. Tau-positive astrocytes are also a major pathological feature of other tauopathies including Pick's disease, corticobasal degeneration, progressive supranuclear palsy and frontal–temporal dementia (FTD), and are also commonly found in the elderly, a condition referred to as aging-related tau astrogliopathy (ARTAG) [15, 28, 29]. In each of these, the distribution as well as the morphology is distinctive to each disease.

Over the course of investigating CTE radiological–pathological correlations as described in Gangolli et al. and Holleran et al. [19, 24], we observed an unusual astrocytic pathology which has not been reported in previous literature regarding CTE to our knowledge. This pathology is characterized by beaded processes and puncta-like structures, typically found in the white matter regions. Additionally, the pathology shows a considerable variation both in severity and distribution across several tissues. We hypothesize that this pathology is representative of degenerating astrocytes within the brain and can be quantitatively distinguished from intact reactive astrocytes. Finally, we have begun to explore the relationship between astrocytic degeneration and tau pathology.

Materials and methods

Tissue samples

A total of 26 randomly selected samples of superior frontal cortex Brodmann Area 8/9 were evaluated (Table 1). Samples 1–9 represented a first cohort and consisted solely of subjects with a confirmed neuropathological diagnosis of stage III or stage IV CTE. Samples 10–19 represented a second cohort that consisted of subjects with a diagnosis of FTD, AD, or CTE. The investigators were blinded to the diagnosis of each of the second cohort subjects when performing the analysis. Sample 20 was an independent tissue sample that was obtained and examined by the Anatomic Pathology Labs at Washington University in St. Louis. This tissue was not evaluated or quantified together with the remaining 25 cases. Finally, samples 21–26 represented 6 age-matched control subjects. With the exception of sample 20, all of the cases were evaluated using identical methodology, with the only differences in whether the investigators were blinded or not. All tissues were obtained from the VA-BU-CLF bank, and neuropathological evaluation was performed through Boston University School of Medicine and Washington University School of Medicine. Next of kin provided written consent for participation and brain donation. All tissue samples were initially stored in periodate–lysine–paraformaldehyde (PLP) fixative.

Immunohistochemistry

Prior to the histopathological analyses reported here, the tissue blocks were imaged using ex vivo MRI. In preparation for MRI excess fixative was washed from the tissue by incubating the tissue blocks in PBS/azide for 2 weeks. Following MRI data acquisition [24], tissues were incubated in 10% formalin and refrigerated at 4 °C for 1 week, and then equilibrated in 30% sucrose for another week. The tissue was then sectioned at 50-µm thickness using a freezing sliding microtome and stored in cryoprotectant. The exception was case 20, which was not prepared for MRI. This tissue was sectioned, paraffin embedded and stained independently by the Anatomic Pathology Labs at Washington University in St. Louis.

Table 1 Case information

Case	Disease	CTE stage	Age	PMI	Comorbidities	ABC score	Astrocyte degeneration	TDP 43	Alpha-synuclein
1	CTE	III	70–79	33 h	Vascular disease (leukoencephalopathy); AD changes	A2B2C1	Positive	0	0
2	CTE	IV	60–69	2.5 h	Vascular disease	A2B2C0	Positive	Hpc, Neoctx	0
3	CTE	IV	70–79	2–10 h	Lewy body disease; vascular disease	A3B2C1	Positive	Amyg, Hpc	Neocortical
4	CTE	IV	80–89	80–92 h	Lewy body disease; vascular disease	A2B0C1[c]	Negative	Amyg, Hpc, EC/inferior temp	Neocortical
5	CTE	IV	70–79	37 h 10 min	Vascular disease	A0B2C0	Positive	Amyg, Hpc, EC/inferior temp	0
6	CTE	III	40–49	43–48 h	None noted	A1B2C0	Positive	0	0
7	CTE	III	80–89	N/K	Hippocampal sclerosis; Lewy body disease	A1B0C0[c]	Positive	Amyg, Hpc, EC/inferior temp	Limbic
8	CTE	IV	60–69	8–20 h	None	N/K	Negative	Not Assessed	Not assessed
9	CTE	IV	80–89	6 h 40 min	Lewy body disease; vascular disease	A3B2C1	Negative[a]	Amyg, EC/inferior temp	Neocortical
10	CTE	IV	70–79	15 h 26 min	Vascular disease; Lewy bodies in olfactory bulb	A3B2C1	Negative	Hpc, EC/inferior temp, Neocortex	OB
11	CTE	III	50–59	10 h 23 min	None noted	A0B2C0	Negative	0	0
12	CTE	III	70–79	12.5 h	Vascular disease	A1B2C0	Positive	Amyg, EC/inferior temp, Neocortex	0
13	FTD	N/A	90–99	10 h	FTLD—tangle-only	A0B2C0	Negative	Brainstem, Hpc, EC, Amyg	0
14	AD	N/A	80–89	4 h 42 min	AD; vascular disease	A2B3C3	Positive	Not assessed	0
15	FTD	N/A	80–89	9 h	FTLD—progressive supranuclear palsy; vascular disease	A1B1C1	Positive	Not assessed	0
16	AD	N/A	90–99	7.25 h	AD; vascular disease	A2B3C3	Positive	Not assessed	0
17	AD	N/A	70–79	4.5 h	AD; vascular disease	A3B3C3	Negative	Not assessed	0
18	FTD	N/A	70–79	14 h 20 min	Picks; AD; vascular disease	A2B2C3	Positive	Not assessed	Brainstem
19	CTE	IV	60–69	9 h 10 min	White matter rarefication	A3B2C2	Positive	Not assessed	0
20	CTE	IV	60–69	N/K	None noted	N/K	Positive[b]	Not assessed	Not assessed
21	Healthy	N/A	40–49	6 h 30 min	None	N/K	Positive	Not assessed	Not assessed
22	Healthy	N/A	50–59	> 24 h	None	N/K	Negative	Not assessed	Not assessed
23	Healthy	N/A	60–69	72 h	None	N/K	Negative	Not assessed	Not assessed
23	Healthy	N/A	70–79	24 h	None	N/K	Positive	Not assessed	Not assessed
25	Healthy	N/A	80–89	22 h	Vascular disease	N/K	Negative	Not assessed	Not assessed
26	Healthy	N/A	90–99	22 h	Vascular disease	N/K	Negative	Not assessed	Not assessed

AD pathology, AB Plaque Score: A0: no AB or amyloid plaques; A1: THAL phases 1 or 2; A2: THAL phase 3; A3: THAL phases 4 or 5, NFT stage B0: no NFTS: B1: Braak stage I or II: B2: Braak stage Iii or IV; B3: Braak stage V or VI, Neuritic Plaque Score: C0: no neuritic plaques; C1: CERAD score sparse; C2: CERAD score moderate; C3: CERAD score frequent

CTE Chronic traumatic encephalopathy, *FTD* frontal–temporal dementia, *AD* Alzheimer's disease, *N/K* not known, *PMI* post-mortem interva, *Amyg* amygdala, *EC/inferior temp* entorhinal cortex/inferior temporal lobe, *Hpc* hippocampus

[a]Displayed degeneration in the gray matter but not the white matter

[b]Tissue section was stained by an independent lab, which indicated astrocyte degeneration but could not be quantified

[c]Pathological examination yielded no signs of NFTS, although extensive CTE-related tauopathy and degeneration may have obscured the AD morphology

Serial tissue sections were assessed for phosphorylated tau pathology [24] or reactive astrocytes. The interval between sections stained for the same marker was 250 µm. Sections were subjected to antigen retrieval, for either 5 mins in 70% formic acid (p-tau) or 3 mins in sodium citrate buffer (pH 6.0) heated to 90 °C (reactive astrocytes). After washing in TBS, sections were incubated in 3% hydrogen peroxide for 10 mins to permeabilize the membrane and washed again in TBS. Non-specific binding was blocked by incubating sections in 3% normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 30 mins, followed by overnight incubation at 4 °C in primary antibody to stain for either p-tau (AT8, Phospho-PHF-tau pSer202 + Thr205 mouse anti-human monoclonal antibody ThermoFisher Scientific) or reactive astrocytes (GFAP, mouse anti-human monoclonal antibody, Millipore, MA, USA) at 1:1000 dilution in blocking serum. Sections were then washed and incubated for 1 h at room temperature in biotinylated goat anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA, USA) diluted 1:1000 in TBS-X. Specific immunoreactivity was detected by using avidin–biotin horseradish peroxidase complex (VECTASTAIN Elite ABC HRP Kit, Vector Laboratories, Burlingame, CA, USA) diluted 1:400 in TBS for 1 h. After washing in TBS, sections were visualized using 3-3′diaminobenzidine DAB (Sigma-Aldrich USA) and mounted onto positively charged slides (Globe Scientific, NJ, USA). Mounted tissue sections were then counterstained using Harris Hematoxylin (Sigma-Aldrich, USA) for 45 s followed by differentiation in 0.25% acid alcohol. All tissue sections were dehydrated using a series of graded ethanols (50%, 70%, 95%, 100%, 100%), cleared in xylene, and cover slipped (Microscope Coverglass No. 1.5, Globe Scientific, NJ, USA).

Image acquisition

Digital images of the sections were acquired using the Zeiss Axioscan Z1 automated Slide Scanning System (Zeiss Microscopy) using a 20× optical magnification. The images were then exported from CZI format to tiff image format using Zen (Zeiss Microscopy), resizing to 30% of the original resolution and exported as mosaic panels. Color deconvolution was then performed on the exported tiffs using the color deconvolution function in FIJI (NIH, Bethesda MD, USA). The 8-bit mosaic panel tiffs were then stitched together using a custom written script (Matlab 2017, Mathworks) generating 8-bit tiffs of the full tissue sections of both the DAB and hematoxylin stains (Supp. Figure 1).

Identification of regions of interest

For each tissue block, regions of interest (ROIs) included total gray matter, gray matter sulcal depths, total superficial white matter, superficial white matter adjacent to sulcal depths, and deep cortical white matter (Supp. Figure 2). All ROIs were drawn using the deconvolved hematoxylin component of stained sections in order to remain blinded to astrocytic and p-tau pathology. For this analysis, superficial white matter was defined as a shell with an outer rim delineated by the gray/white matter boundary, and the inner rim extending 1 mm into the white matter. Remaining white matter was classified as deep cortical white matter. A sulcal depth was defined as the bottom two-thirds of connecting gyri, extending into the gray matter from the gray/white matter boundary. White matter adjacent to sulcal depths was defined as a sub-region of superficial white matter, extending from boundary of the sulcal depth ROI to 1 mm into white matter.

Degenerating astrocyte quantification

Each of the tissues was analyzed quantitatively utilizing FIJI. The percent area of degenerating astrocytes in white matter was quantified using automatic thresholding in ImageJ based on intensity of DAB staining (Supp. Figure 3) [24]. Each individual image was thresholded separately as the DAB staining varied slightly between tissue samples. To ensure reproducibility of the thresholding, a second investigator thresholded 10 individual tissue images. These 10 images were then processed using the same quantitative methods and showed strong correlation ($r = 0.96$, $p < 0.0001$) (Supp. Figure 4). Size inclusion was set at 0–50 pixels and solidity was set at 0.7–1.0. These values were obtained through testing of various parameters and determining the values that best captured the majority of the degenerating astrocytes but did not include non-degenerating reactive astrocytes (Supp. Figure 5). Non-degenerating reactive astrocytes typically had a larger size and a lower solidity compared to the beaded structures that characterized the degenerating astrocytes. Particle analysis was carried out using the "Extended Particle Analyzer" tool in the BioVoxxel toolbox [50].

Total reactive astrocyte percent area was assessed with size inclusion of 0–10,000 pixels in the white matter to include full-sized non-degenerating astrocytes, but exclude larger non-specific immunoreactive structures. The same gray and white matter ROIs used to quantify degeneration were used for quantifying total astrocyte percent area.

Within the gray matter, astrogliosis tended to be denser and tightly clustered. As a result, thresholding of astrocytes in the gray matter identified larger, connected astrocytic structures rather than individual astrocytes. To take this into account, the size inclusion parameters for gray matter astrocytes were set to 0–20,000 pixels rather than 0–10,000.

Quantification of phosphorylated tau pathology

The deconvolved DAB component of AT8-stained sections was used to quantify p-tau pathology. AT8-positive staining was automatically thresholded in FIJI using the Sauvola local thresholding method (radius = 50 pixels) followed by a size inclusion criterion of 50–10,000 pixels to exclude out-of-focus structures and artifacts such as tissue edges and tearing. The ratio of percent area of p-tau staining in each sulcal depth to percent area p-tau staining in gray matter was used to quantify the localization of tau pathology in cortical sulci.

Statistical analyses

Statistical analyses were performed in R. Spearman rank correlations were used to examine the relationship between astrocytic degeneration, white and gray matter astrogliosis, sulcal astrogliosis, and tau aggregation. A one-way ANOVA was used to examine the relationship between astrocytic degeneration and age. Student's t tests were performed to analyze differences between grades of CTE. Graphs were created using Prism.

Results

All CTE, AD, and FTD brain samples displayed astrogliosis

All tissue samples were positive for reactive astrocytes as reflected by GFAP immunohistochemistry. However, there was substantial variability in the extent of astrocytic immunoreactivity between brain samples and across anatomical boundaries (Fig. 1). Astrogliosis was most commonly present within the gray-white matter junction but was also present in the deeper white matter as well as gray matter. There appeared to be a quantitative difference between disease states in the extent of gray matter astrogliosis (Fig. 2a), with lower levels of astrogliosis in CTE brain samples compared to FTD brain. However, overall white matter astrogliosis did not appear to vary by disease or stage of CTE (Fig. 2b). We examined the extent of astrogliosis present within the sulcal depths, as well as in the white matter adjacent to the sulcal depths. We found that sulcal astrogliosis appeared to be lower in CTE subjects compared to both FTD and AD subjects (Fig. 2c). Additionally, the ratio of sulcal astrogliosis to overall astrogliosis was higher in FTD and AD cases compared to CTE cases (Fig. 2d). These results indicate that astrogliosis in CTE subjects may be more diffuse across the entire tissue, while in FTD and AD, astrogliosis is concentrated within the sulcal regions. The healthy controls displayed varying levels of astrogliosis, ranging from just a few astrocytes along the tissue edges to significant astrogliosis in the gray–white matter junction (Supp. Figure 6a). However, they displayed very little astrogliosis within the deep white matter, particularly compared to the disease tissue (Fig. 2b.)

A subset of CTE, AD, and FTD brain samples had apparent astrocytic degeneration

In addition to GFAP-stained cells with the morphology of typical astrocytes (Fig. 3a), we also observed what appeared to be extensive degenerating astrocyte pathology, typically characterized by beaded, puncta-like GFAP immunoreactivity (Fig. 3b–d). This apparent astrocytic degeneration was identified in 13 out of 20 cases, including 9/13 CTE cases, 2/3 AD cases and 2/3 FTD cases (Table 1). Additionally, 2 of 6 healthy controls displayed evidence of degeneration, although both of these displayed lower levels of degeneration than any of the disease cases (0.29% and 0.18%, whereas the lowest amongst the disease cases was 0.31%). Additionally, one of those two cases had an extent below what our quantitative method was capable of detecting. The degree of degeneration varied substantially between individual tissues, ranging from modest, with just a few degenerating astrocytes (Fig. 3b), to extensive with entire fields dominated by GFAP immunoreactive punctae (Fig. 3d). This astrocytic degeneration was typically located within the white matter, although there was evidence of gray matter involvement in 2 of the 13 disease cases positive for astrocytic degeneration (Supp Fig. 7), as well as in one healthy control. In both of these disease cases, degeneration was prominent near the edges of the tissue, although neither case exhibited extensive amounts of degeneration. Additionally, in one of these gray matter degeneration cases, there was no astrocytic degeneration apparent within the white matter. We did not discern stereotypic locations within the white matter where the astrocytic degeneration was concentrated. Typically, tissues which exhibited extensive astrocytic degeneration in the white matter also displayed extensive non-degenerating GFAP immunoreactive astrocytes in both the white matter and the gray matter regions, and non-degenerating astrocytes commonly co-localized with degenerating astrocytes (Fig. 4). In the 7 cases without white matter degeneration, there was typically little to no GFAP-positive staining in the white matter.

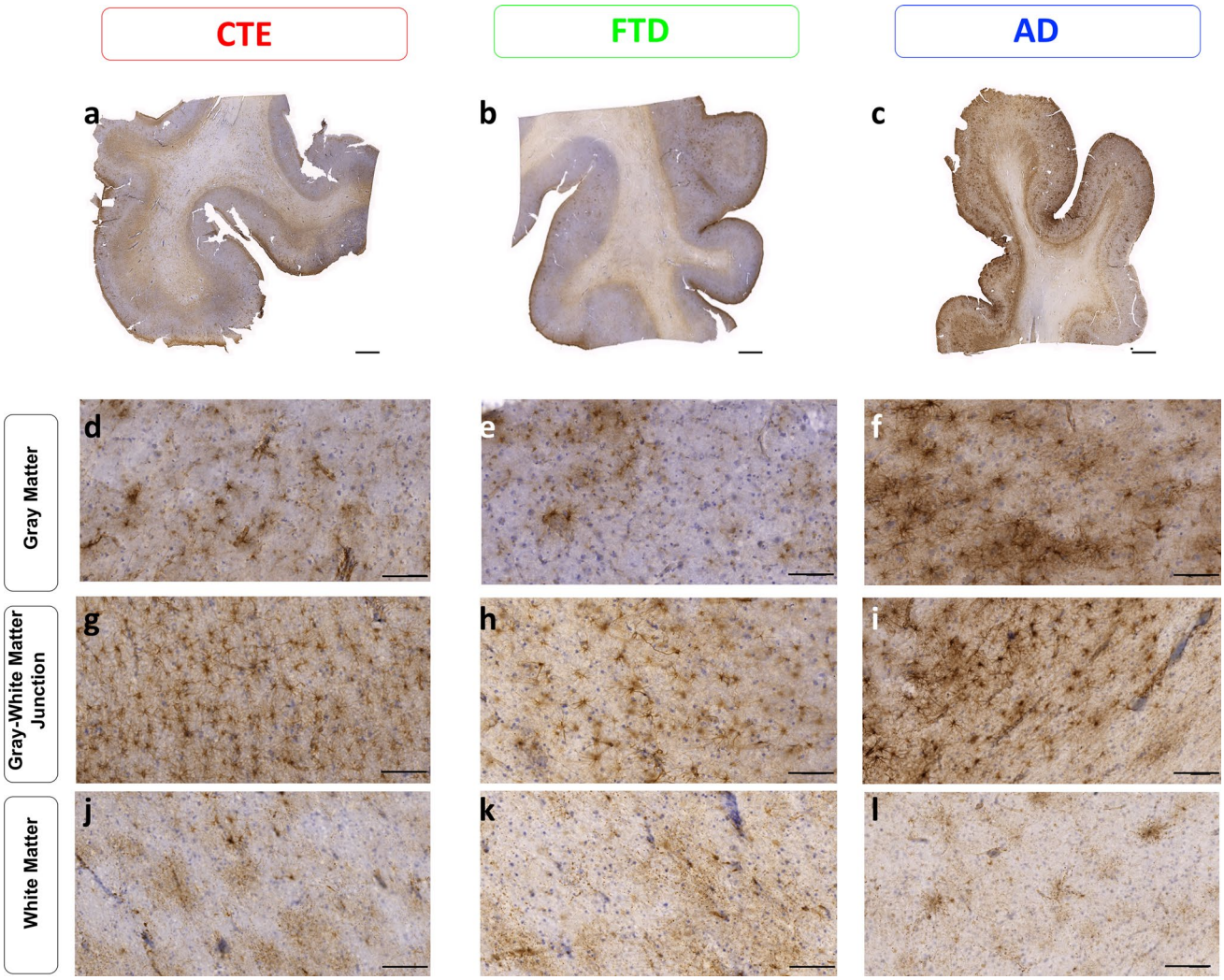

Fig. 1 Representative GFAP immunohistochemistry demonstrates astrogliosis in patients with CTE, AD, FTD. **a–c** GFAP immunohistochemistry in frontal cortex of CTE (case 12), FTD (case 18), and AD (case 14), respectively. **d–f** GFAP immunohistochemistry demonstrating stereotypical astrogliosis in the gray matter. **g–i** GFAP immunohistochemistry in gray–white matter junction. **j–l** GFAP immunohistochemistry in white matter. Scale bars **a–c** 2000 µm. **d–l** 100 µm

Astrocytic degeneration did not appear to be correlated with disease

We analyzed the relationship between white matter astrocytic degeneration and disease (Fig. 5a) and found there did not appear to be a quantitative difference. Additionally, the extent of white matter astrocytic degeneration found in stage III CTE vs. stage IV CTE was not significantly different (Student's t test, $p = 0.54$). Additionally, there was no relationship between disease and the extent of degeneration in the superficial or deep cortical white matter (Fig. 5b, c). We examined the ratio between astrocytic degeneration found in the superficial white matter compared to deep cortical white matter with respect to disease and found the average ratio was 1.2 ± 1.0 and did not appear to vary between diseases (Fig. 5d), suggesting that astrocytic degeneration is diffuse across the entire white matter rather than being concentrated in either the superficial white matter or the deep cortical white matter. Because overall astrogliosis was quantitatively higher in the sulcal depths of FTD and AD cases compared to CTE cases (Fig. 2), we examined the extent of astrocytic degeneration specifically in the superficial white matter adjacent to the sulcal depths, and found that there did not appear to be a quantitative difference between diseases (Fig. 5e).

Vascular disease, which is commonly found in cases of neurodegeneration, was a common co-morbidity, which is not unexpected due to the severity and age of many of our cases (Table 1). However, it should be noted that astrocytic degeneration was present in 8 of 15 cases with vascular

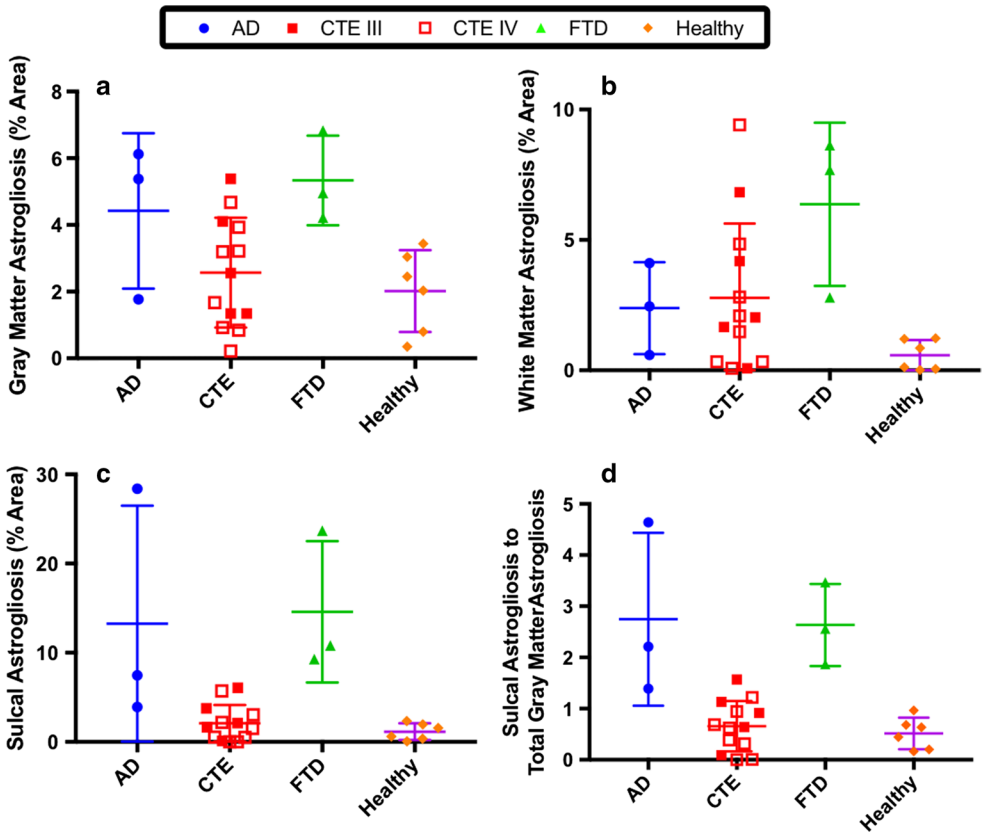

Fig. 2 Extent of astrogliosis based on disease. **a** Gray matter astrogliosis appeared higher in FTD subjects compared to CTE patients or healthy controls. **b** Total white matter astrogliosis appeared higher in FTD subjects compared to CTE patients or healthy controls. **c** There were substantially higher levels of astrogliosis within the sulcal depths of AD and FTD subjects compared to CTE subjects or healthy controls. **d** There was a higher ratio of sulcal astrogliosis to total gray matter astrogliosis in AD and FTD than in CTE or healthy controls. Each symbol represents mean value for one tissue sample

disease and 6 of 11 cases without vascular disease, showing that astrocytic degeneration did not appear to be related to the presence of vascular disease.

Astrocytic degeneration did not appear to be an artifact

We performed multiple controls to address the possibility that the apparent astrocytic degeneration was an artifact of the methods used. Negative controls involving exclusion of the primary antibody in a CTE brain showed no staining (Supp. Figure 8a). The full GFAP immunohistochemistry protocol in a brain without known neuropathology showed no staining under identical conditions (Supp. Figure 8b). To assess whether this degeneration was specific to the anti-GFAP monoclonal antibody used, we performed a subsequent staining with a different, polyclonal anti-GFAP antibody. We were able to identify qualitatively similar astrocytic degeneration within the tissue, albeit with significantly more background staining (Supp. Figure 8c). Additionally, a separate tissue block from the VA-BU-CLF bank was stained independently by the Anatomic Pathology Labs at Washington University in St. Louis and yielded similar astrocytic degeneration (Fig. 4, Supp. Figure 8d). This block had not been processed for MRI prior to immunohistochemistry and was embedded in paraffin with 10 micron sections rather than cut on a freezing sliding microtome with 50 micron sections. We found no relationship between astrocytic degeneration with age by decade ($F_{(5,13}=0.31$, $p=0.90$; Fig. 6a), and there was no correlation evident between the extent of the astrocytic degeneration and the post-mortem interval ($r_s=-0.11$, $p=0.64$; Fig. 6b). Additionally, the two healthy controls that demonstrated degeneration were in their 40s and 60s, and our older cases showed no signs of degeneration, suggesting that the pathology is not a result of simple aging.

Astrocytic degeneration was correlated with overall astrogliosis

Quantitatively, the extent of astrocytic degeneration was strongly correlated with the extent of total astrogliosis, both in white matter and the gray matter (Fig. 7a, b). Additionally, there appeared to be a quantitative difference between the ratio of astrocytic degeneration in the white matter to total white matter astrogliosis between CTE other cases, with the highest ratio present in CTE cases (Fig. 7c).

There was a modest but significant correlation between sulcal astrogliosis and the degeneration in the corresponding adjacent white matter region (Fig. 8a, $r_s=0.57$, $p=0.00037$). The overall astrocytic degeneration across the entire white matter was also correlated with the extent of gray matter

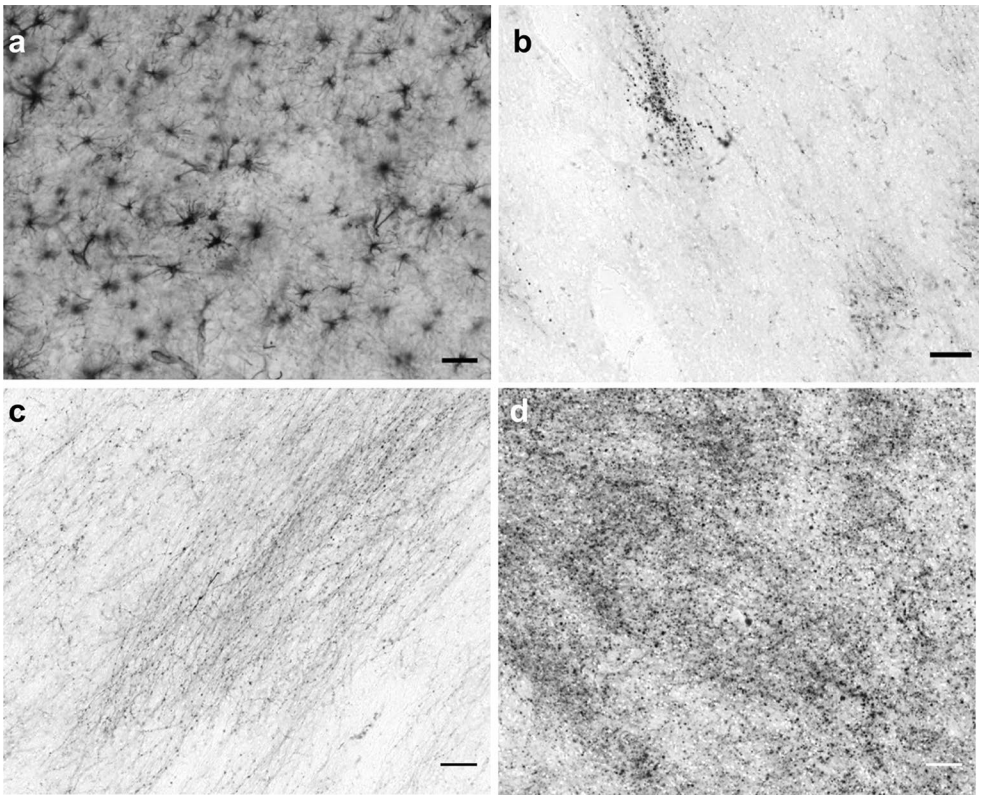

Fig. 3 Varying levels of astrocytic degeneration in CTE. **a** Non-degenerating astrocytes found in the white matter (case 1). **b** A single astrocyte exhibiting characteristic beaded processes of degeneration (case 2). **c** Beads-on-a-string pathology (case 3). **d** Widespread astrocytic degeneration throughout the white matter in the most severe case in the series (case 5). No discernable intact astrocytic structures remaining. All images were stained for GFAP and hematoxylin then color deconvolved to isolate DAB staining. Scale bars 50 µm

sulcal astrogliosis (Fig. 8b, $r_s = 0.76$, $p = 0.0001$) and astrocytic degeneration in the white matter adjacent to the sulcal depths (Fig. 8c, $r_s = 0.62$, $p = 0.0043$).

Astrogliosis and astrocytic degeneration are not correlated with tau aggregation

Each of our CTE, AD, and FTD cases displayed p-tau pathology consistent with pre-existing literature (Fig. 9), and we examined the relationship between astrocytic degeneration, astrogliosis, and tau aggregation. Overall, we found no significant correlations between tau pathology and astrogliosis or astrocytic degeneration. White and gray matter astrogliosis were not correlated with tau aggregation, nor was white matter degeneration (Fig. 10a–c). Additionally, the extent of sulcal astrogliosis, as well as astrocytic degeneration in the white matter adjacent to cortical sulci, were not correlated with tau aggregation on a sulcus by sulcus basis (Fig. 10d, e). We observed morphological characteristics consistent with degenerating astrocytes in some p-tau immunostained sections (Supp. Figure 9). However, our quantitative p-tau analysis was not exclusively focused on astroglial tau, and we have not performed double immunolabeling to definitively assess the relationship between p-tau pathology and astrocytic degeneration at the single cell level.

Discussion

In summary, we quantitatively examined the GFAP immunopathology in cortical brain tissues from CTE, AD, and FTD patients. A new finding was a degenerating astrocyte pathology in CTE that was observed in approximately two-thirds of the cases. The degeneration was characterized primarily by beaded GFAP immunoreactive processes, typically located in the white matter. In the most extreme case, the entirety of the white matter was laden with bead-like GFAP immunoreactive structures, with no stereotypical astrocytes identifiable. The extent of astrocytic degeneration was highly correlated with increased levels of overall reactive astrogliosis both in the white and gray matter and was similar in both superficial and deep cortical white matter. The extent of astrocytic degeneration was not related to the subjects' ages, disease, and in the case of CTE subjects, the stage of CTE. Further, while CTE is characterized by hyperphosphorylated tau aggregation

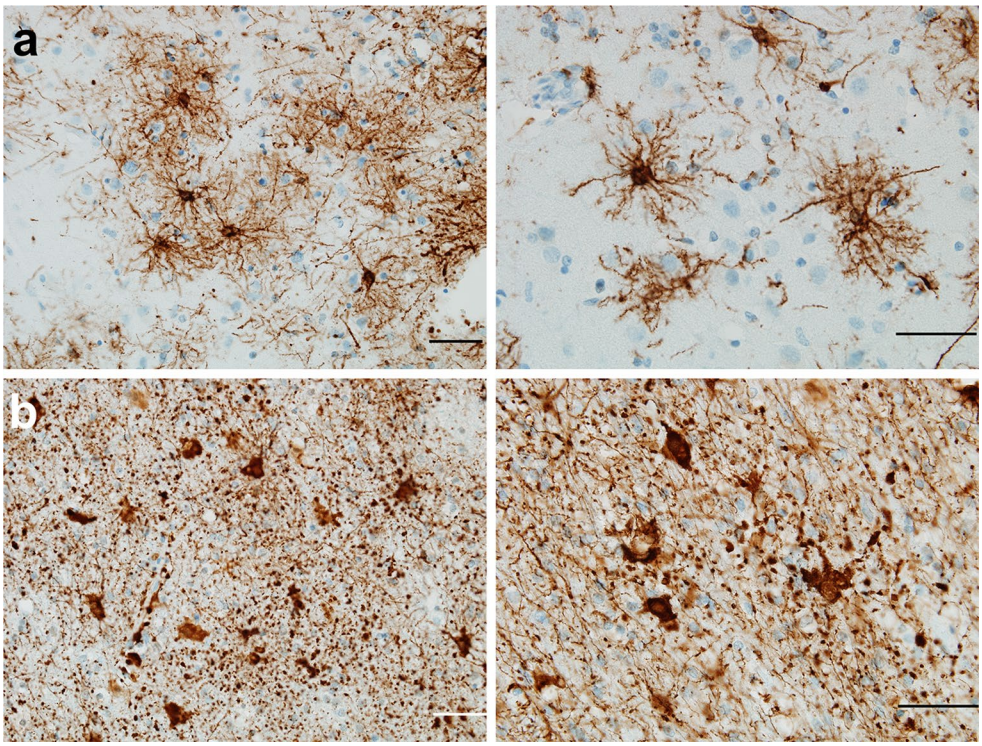

Fig. 4 Astrocytosis and astrocytic degeneration in thin sections from an independent lab. **a** Stereotypical, non-degenerating astrogliosis in the gray matter of a CTE confirmed case (case 20) (left, low magnification; right, high magnification). **b** Degenerating astrocytes with swollen end processes in the white matter of the same CTE case. Scale bars 250 µm

within the gray matter sulcal regions, we found no correlation between either astrogliosis or astrocytic degeneration and p-tau aggregation. We did find that astrogliosis in CTE subjects was diffuse, while astrogliosis in both AD and FTD patients was concentrated within the sulcal depths. This degeneration was also found in two of our healthy cases, although in minimal amounts, suggesting that there may be multiple pathways that result in the degeneration of astrocytes.

There is no existing literature to our knowledge that reports on degenerating astrocytes in CTE subjects. However, the observed astroglial pathology resembles those found in some blast-TBI patients reported in Shively et al. [51]. The pathology also bears a resemblance to the astrocytic degeneration found in patients with neuromyelitis optica (NMO), an autoimmune disorder where autoantibodies against aquaporin 4 (AQP4) appear to trigger astrocytic degeneration [9, 47]. Previous studies have reported the presence of astrocytic degeneration in FTD and AD patients, albeit sparingly [31, 62]. Tomimoto et al. found that astrocytic degeneration (which they termed clasmatodendritic astrocytes) was present in white matter lesions of select dementia patients, and those patients were also positive for complement antibodies like C3d [62]. Higher levels of astrocytic apoptosis have been correlated with more severe disease stages in FTD [8, 31].

Limitations

For this study, we analyzed only stage III and IV CTE tissue samples. Analysis of stage I and II tissue will be necessary to determine whether astrocytic degeneration occurs at earlier stages of CTE and in younger individuals. Additionally, there were small sample sizes for FTD and AD groups, and thus it was not possible to make concrete statistical comparisons between disease states. Larger sample sizes may reveal subtle differences between diseases that may not have been detected in the current study. Our study was also limited to frontal cortical regions; further analysis of deeper white matter tissues, deeper gray matter structures and other cortical regions will be needed to determine the true extent of astrocytic degeneration.

Currently, we have only used GFAP to mark reactive astrocytes. Further analysis with additional markers like S100β and aquaporin 4 would be appropriate for future investigations. It was also unclear whether degenerating astrocytes were p-tau-positive or not, as our tissues were single labeled for either GFAP or p-tau, rather than double labeled. Previous descriptions of astrocytic tau have

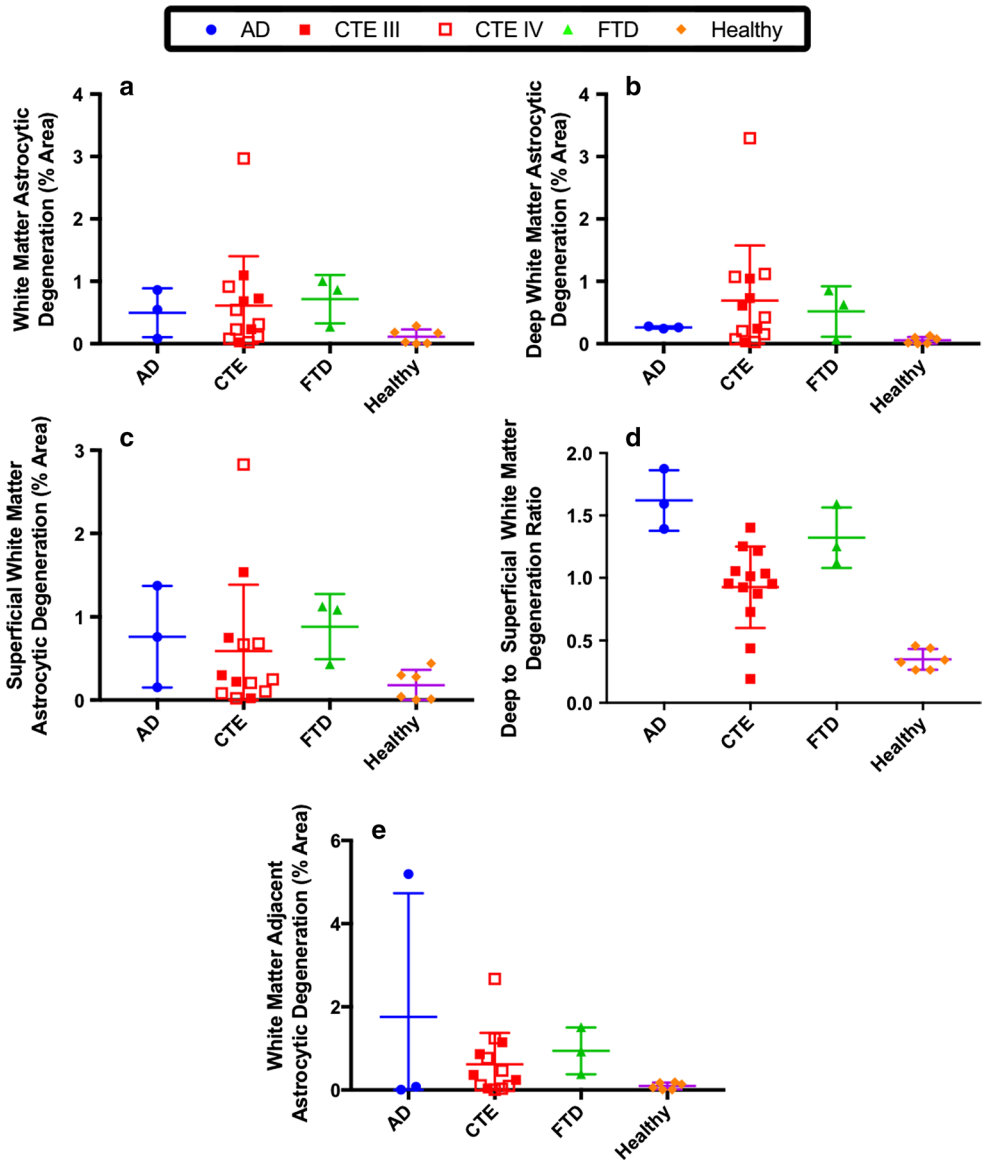

Fig. 5 White matter degeneration based on location and disease. **a**–**c** Total white matter astrocytic degeneration, deep white matter astrocytic degeneration, as well as superficial white matter astrocytic degeneration did not appear to differ between diseases. **d** The ratio between degeneration found in the superficial white matter and the deep white matter were not quantitatively different between diseases. **e** Astrocytic degeneration in the white matter adjacent to the sulcal depth was different based on disease. Each symbol represents mean value across tissue slices for an individual subject. Vertical error bars represent standard deviations

indicated more typical, non-degenerating reactive astrocyte pathology, suggesting that most degenerating astrocytes are unlikely to be tau-positive [33].

There were also limitations in the quantification of degeneration using FIJI due to the heterogeneity of reactive and degenerating astrocytes. Degenerating astrocytes were characterized by beaded processes and, in the most severe cases, by puncta-like staining. However, some reactive astrocytes within the white matter featured small processes which, when thresholded, yielded a circular structure not unlike those of degenerating astrocytes. To counteract this, we were forced to use smaller size criteria for the punctate staining that characterized the astrocytic degeneration, which may have led to a systematic undercounting of the extent of astrocytic degeneration. Additionally, for some astrocytes only certain portions of the processes were in focus in the images. This caused the thresholding to break the processes occasionally into structures that resembled astrocytic degeneration. This problem was most prevalent within the gray matter, where reactive astrocytes had longer, denser regions of processes. This ultimately prevented us from quantitatively analyzing the extent of astrocytic degeneration in gray matter. However, as noted, astrocytic degeneration was most prominent in white matter. Investigations using confocal microscopy and 3-dimensionally resolved image stacks may be helpful for future quantitative investigations of astroglial pathologies. Stereological methods could also serve as an alternative, unbiased approach, but these methods are extremely time-consuming when dealing with large-sampling tissues.

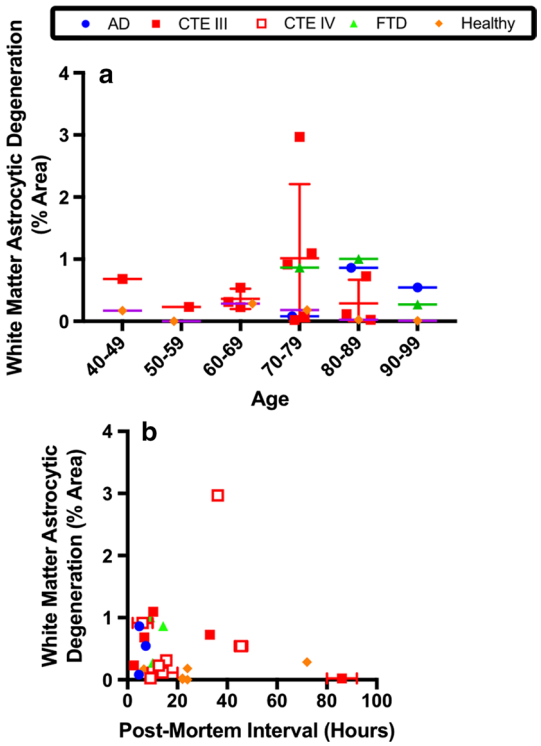

Fig. 6 Analysis of astrocytic degeneration based on age and PMI. **a**, **b** Astrocytic degeneration in the white matter was not correlated with the age of the subjects or the post-mortem interval. Horizontal error bars indicate PMI range when PMI was not known with certainty. Each symbol represents mean value across tissue slices for an individual subject. Vertical error bars represent standard deviations

Future directions

Studies have recently suggested that the mechanism of injury to the brain might be the causative factor in determining whether reactive astrocytes have neurotoxic or neuroprotective properties following astrogliosis. Reactive astrocytes elicited by lipopolysaccharide injections, termed A1 astrocytes, have been reported to have neurotoxic properties, while astrocytes elicited by middle cerebral artery occlusion, termed A2 astrocytes, have been reported to have neuroprotective properties [30, 67]. These two classes of astrocytes appear phenotypically distinct and may help distinguish the role that astrocytes play in neurodegeneration and brain trauma. Other studies have also found that different populations of astrocytes can preferentially express different receptors and ion channels [22, 32]; it is possible that there may be more than two classes of reactive astrocytes that have yet to be reported, similar to the multiple phenotypes found in microglia [43]. Determining the phenotype of the reactive and degenerating astrocytes found in CTE patients, as well as the functional implications of the astrocytic degeneration, could provide valuable insight into understanding the mechanisms of disease in CTE and other neurological disorders characterized by astrocytic degeneration.

An area of particular interest is the mechanism behind the astrocytic degeneration, which is still unknown. Additional studies will be necessary to elucidate whether the degeneration is apoptotic, autoimmune-mediated, or due to another process. Such studies could potentially utilize immunohistochemical markers such as caspase-3 or C3 complement. The degeneration seems unlikely to be a result of direct trauma, as astrocytic degeneration was seen in AD and FTD brains as well as CTE brains. It will also be of interest to determine whether the degeneration predominantly occurs in the white matter because of a localized trigger or because white matter astrocytes differ phenotypically from gray matter astrocytes, causing them to be more vulnerable to degeneration. Studies in FTD patients have found that degenerating astrocytes also express cleaved caspase-3, which indicates a possible apoptotic mechanism [8]. However, other studies have reported GFAP autoantibodies present in the serum of chronic severe TBI patients [64], suggesting that the degeneration could occur in an autoimmune fashion similar to that found in NMO. Increased levels of serum autoantibodies to GFAP as well as other astrocytic proteins like S100β have also been described in AD patients [21, 60, 66]. However, we have not been able to assess serum from the patients whose brains were analyzed in this study. Such paired biofluid and pathological analyses will be important areas for future investigation.

While we report no relationship between astrocyte degeneration and p-tau aggregation, further studies into the morphology and phenotype of p-tau astrocytes still need to be performed to better understand their relationship. We have not detected apparent perivascular distributions of reactive or degenerating astrocytes, but quantitative studies examining the relationship between astrocytic degeneration and vascular structures have yet to be performed. Previous studies have reported axonal disruption present in CTE subjects [19, 24, 35], and determining whether this disruption is correlated with astrocytic degeneration will be of interest. Additionally, because NMO is characterized by demyelination of the optic nerve and the spinal cord and studies have found that astrocytic degeneration precedes myelin loss in NMO patients [48], it will be important to examine the relationship between myelination and astrocytic degeneration in CTE patients. Previous studies have found evidence of myelin loss in AD patients, which supports this possibility [25]. However, because there are currently no validated animal models for CTE, direct mechanistic investigations into these relationships will be difficult.

As there are currently no verified ways of detecting CTE other than post-mortem histopathological analysis, an area of critical importance will be to develop methods to detect astrogliosis and astrocytic degeneration in living patients.

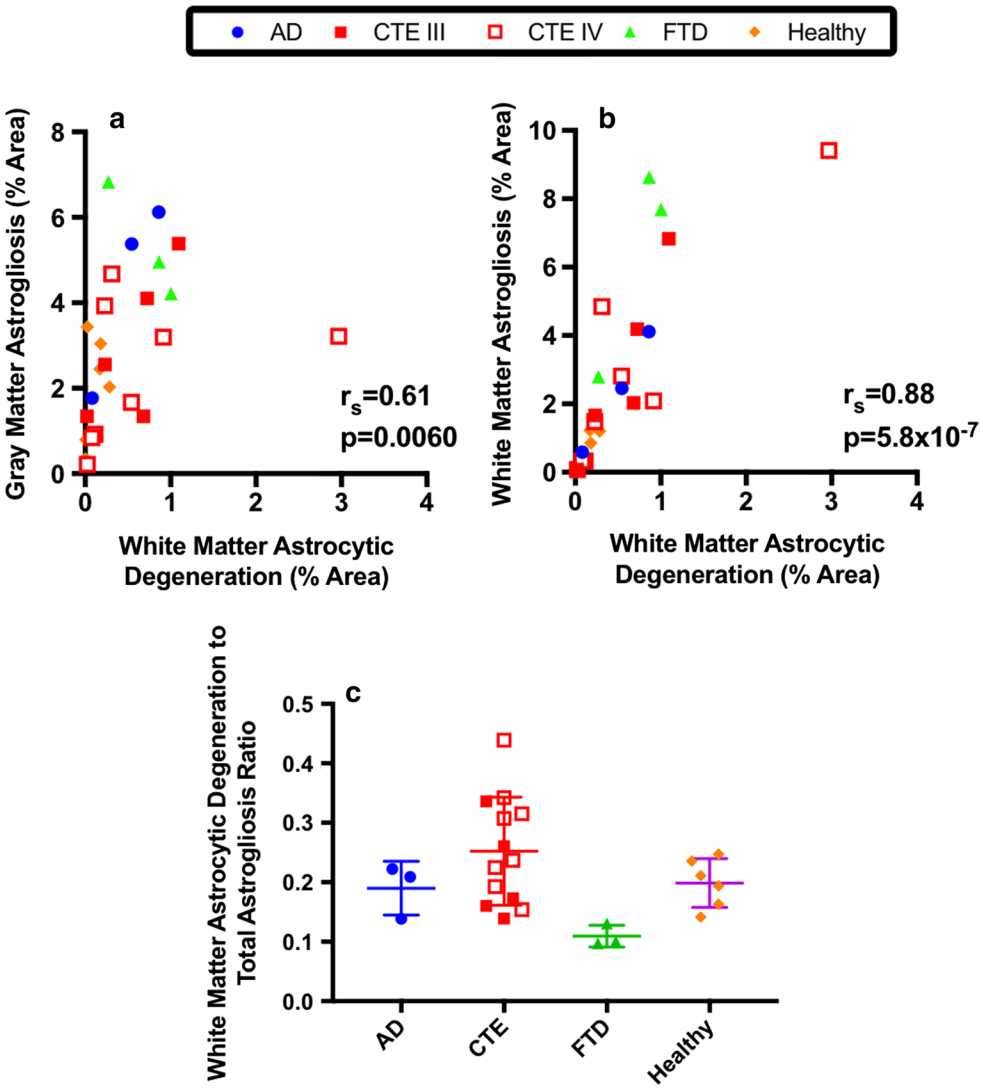

Fig. 7 Quantitative correlations of astrocytic degeneration parameters. **a**, **b** White matter astrocytic degeneration was strongly correlated with total astrogliosis in both the gray and white matter. **c** The ratio of astrocytic degeneration in the white matter to total astrogliosis in the white matter was higher in CTE subjects compared to FTD subjects. Statistical analyses were performed using Spearman rank order correlations. Each symbol represents the mean value for one tissue sample. Vertical error bars represent standard deviations

One possibility will be to determine whether astrogliosis and astrocytic degeneration correlate with any blood or biofluid markers. Alternatively, the ability to detect astrocyte degeneration using advanced imaging would be valuable. Existing studies using PET tracers and high-resolution diffusion MRI techniques have been only modestly successful in detecting neurofibrillary aggregates or axonal degeneration, respectively [6, 19, 24, 44], and it is possible that novel PET ligands and high spatial resolution quantitative MRI techniques can be utilized in the future to detect astrocytic degeneration in CTE patients.

While we did not have information regarding the clinical symptoms of the subjects that we studied, the relationship of astrocytic degeneration and the clinical presentation is an area of great interest. Current studies have suggested that the progression of tau pathology and stage of CTE are more closely correlated with the duration of exposure to rTBI, rather than severity of symptoms [55], suggesting that tau pathology may not be the driving factor behind clinical symptoms. Studies examining the timeline of astrocytic degeneration utilizing both acute and chronic rTBI patients, as well as single TBI patients, will be critical.

Implications

Recent studies have found that astrocytes play a major role in synaptic communication through intracellular calcium changes as well as through the secretion and uptake of gliotransmitters such as glutamate, GABA, and ATP [12, 22, 53]. We speculate that the degeneration of astrocytes could affect synaptic transmission and plasticity, which could lead to behavioral abnormalities such as depression or memory loss. This is supported by the fact that astrocytic degeneration is correlated with more severe disease progression in FTD patients [8]. Additionally, studies have found that patients with depression have fewer astrocytes

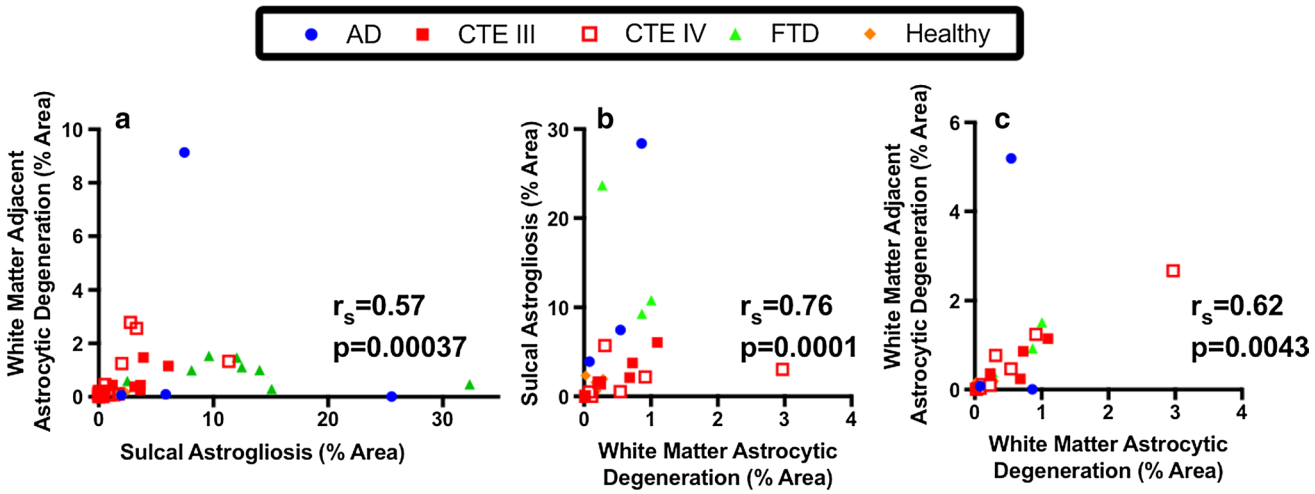

Fig. 8 Astrocytic degeneration in the white matter adjacent to cortical sulci. **a** Gray matter sulcal astrogliosis was modestly correlated with astrocytic degeneration within the white matter adjacent to the sulcus. Each symbol represents an individual sulcus. **b** Total white matter astrocytic degeneration was correlated with gray matter sulcal astrogliosis. **c** Total white matter astrocytic degeneration was correlated with astrocytic degeneration in the white matter adjacent to the sulcal depths. Statistical analyses were performed with Spearman's rank order correlations. In **b**, **c**, each symbol represents mean value for an individual subject

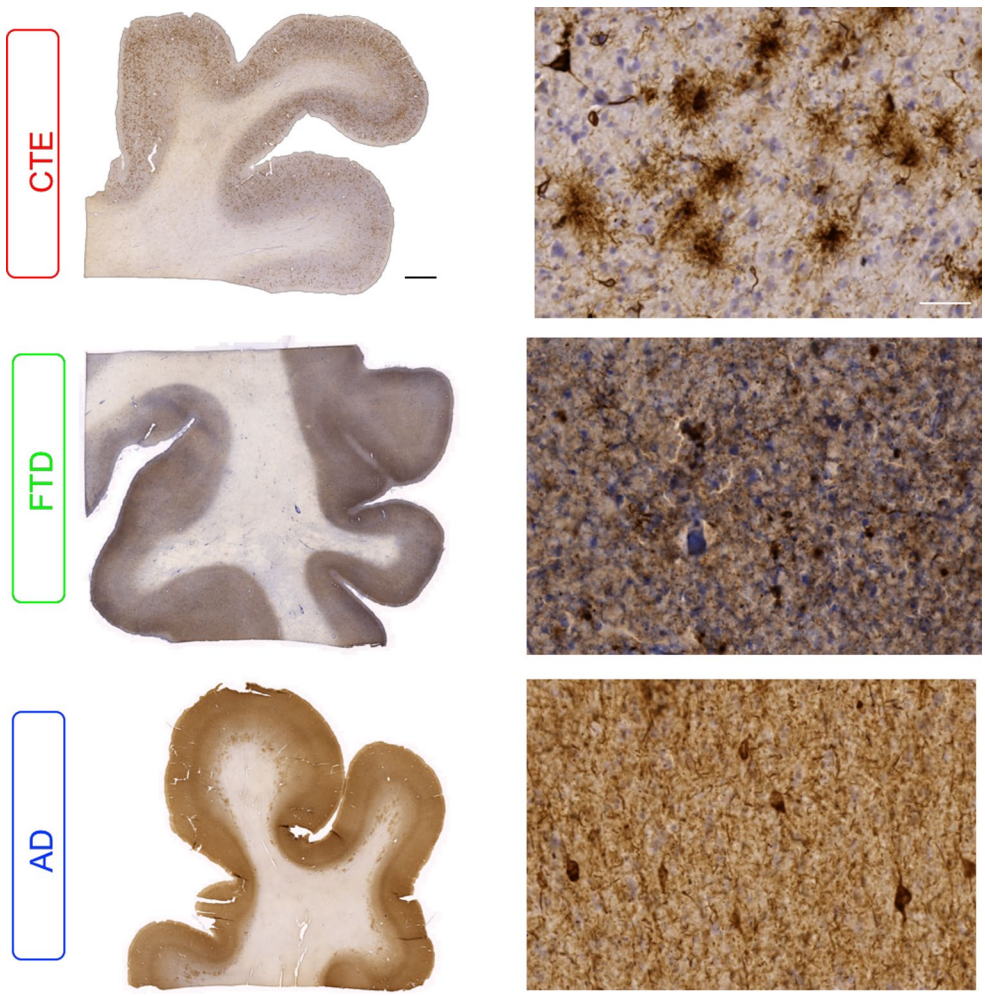

Fig. 9 Tau aggregation in CTE (case 12), FTD (case 18), and AD (case 14). 50-µm-thick sections were stained for p-tau using AT8. Counter staining was performed using Harris hematoxylin. Low power scale bar = 2 mm. High power scale bar = 50 µm

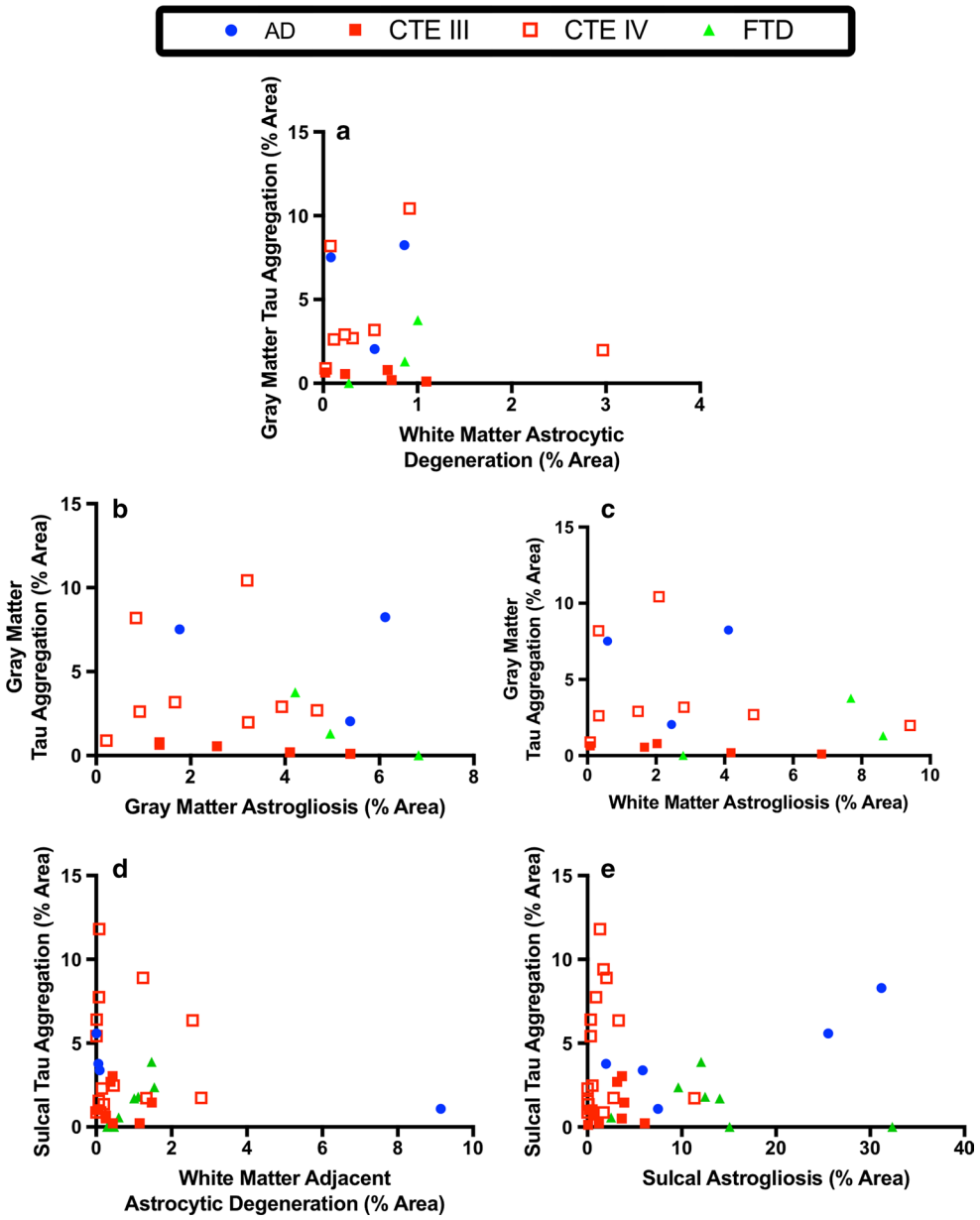

Fig. 10 Astrocytic degeneration and astrogliosis were not correlated with tau aggregation. **a** White matter astrocytic degeneration was not correlated with gray matter p-tau aggregation. **b**, **c** White matter and gray matter overall astrogliosis were not correlated with p-tau aggregation. **d** Astrocytic degeneration in the white matter adjacent to the sulcal depths was not correlated with gray matter sulcal p-tau aggregation ($r_s = 0.027$, $p = 0.54$). **e** Overall astrogliosis in sulcal gray matter was not correlated with sulcal tau aggregation ($r_s = -0.11$, $p = 0.54$). In **a**–**c**, each symbol represents 1 tissue sample. In **d**, **e**, each symbol represents 1 sulcus

compared to healthy controls [22, 49], and other works report that astrocytes play a role in long-term memory consolidation as well as long-term potentiation. [58]. These support the idea that the degeneration of astrocytes in CTE patients may play a role in the onset and progression of clinical symptoms.

Astrocytes are also hypothesized to play a role in the regulation of the blood–brain barrier (BBB) [5, 53, 57]; prior studies have found that astrocytes secrete or express signaling molecules such as Sonic Hedgehog that regulate the BBB through the maintenance of BBB tight junctions [2, 40, 42]. Additionally, astrocytes express angiotensinogen, the loss of which has been shown to cause leaky BBB in mice [42, 65]. It is possible that the degeneration of astrocytes could affect the integrity of the BBB, which could lead to increased concentrations of anti-GFAP autoantibodies entering the brain, if such antibodies are present in the serum. This could lead to a positive-feedback mechanism where, after an initial brain trauma or other insult, degeneration of astrocytes leads to degradation of the BBB, which leads to further degeneration of astrocytes. This is partially supported by an existing study that reports BBB dysfunction in CTE patients by studying p-tau accumulation and claudin-5. However, it is unclear what role astrocytes play in this dysfunction [16]. Studies on the BBB in AD patients are inconclusive, with some studies finding no evidence of leakage, while others have shown impaired barrier function. Furthermore, it is unclear whether any potential BBB

disruption in those patients occurs due to normal aging or specific neurodegenerative processes [11, 17, 54]. Methods to detect astrocytic degeneration in vivo could allow tests of candidate therapeutics targeting this pathology in CTE and other neurodegenerative disorders.

Conclusion

CTE remains a relatively understudied disease with much left to be uncovered about its cause, underlying pathophysiological mechanisms, and clinical–pathological correlations. Our findings suggest that astrocytes degenerate in subjects with CTE, providing additional insight into the pathogenesis of the disease and its origin.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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