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Abstract

Objective: To test the hypothesis that the nucleus basalis of Meynert (nbM), a cholinergic basal forebrain (CBF) cortical projection system, develops neurofibrillary tangles (NFTs) during the progressive pathological stages of chronic traumatic encephalopathy (CTE) in the brain of athletes.

Method: To characterize NFT pathology, tau-antibodies marking early, intermediate and late stages of NFT development in CBF tissue obtained at autopsy from eighteen former athletes and veterans with a history of repetitive mild traumatic brain injury (TBI) were used.

Results: Analysis revealed that cholinergic nbM neurons develop intracellular tau-immunoreactive changes progressively across the pathological stages of CTE. In particular, there was an increase in pre-tangle (phosphorylated pS242) and oligomeric (TOC1 and TNT1) forms of tau in stage IV compared to stage II CTE cases. The nbM neurons also displayed pathologic TDP-43 inclusions and diffuse extracellular and vascular amyloid-β (Aβ) deposits in CTE. A higher percentage of pS242/pT517, pS242 and TNT1 labelled neurons were significantly correlated with age at symptom onset, interval between symptom onset and death and age at death.

Conclusion: The development of NFTs within the cholinergic nbM neurons could contribute to an axonal disconnection in CTE. Further studies are needed to determine the mechanism driving NFT formation in the nbM neurons and its relation to chronic cognitive dysfunction in CTE.

Introduction

Traumatic brain injury (TBI) is the signature wound of recent military conflicts, with 20% of US service men and women sustaining at least one head injury, mainly mild TBI [1,2]. TBI is associated with onset of long-term behavioural and cognitive problems [3,4], but the underlying neurobiological mechanisms remain unclear. Military personnel in the battlefield and athletes in contact sports including boxing and American football [5–12] are exposed to mild repetitive TBI, which can result in chronic traumatic encephalopathy (CTE). The neuropathology of CTE is characterized by intracellular accumulation of abnormally phosphorylated tau protein (p-tau), the main constituent of neurofibrillary tangles (NFT) in Alzheimer’s disease (AD) and non-AD tauopathies [13]. While neuropathological investigations have demonstrated extensive cortical damage in CTE [14], the potential involvement of sub-cortical neurotransmitter systems is poorly understood. Among the sub-cortical systems displaying NFTs in AD are cholinergic neurons of the basal forebrain which provide the major acetylcholine (Ach) innervation to the cortical mantle and hippocampus and are involved in memory and cognitive functions [15–18]. The cholinergic neurons within the basal forebrain are contained within several sub-fields extending rostrally from the septal diagonal band throughout the nucleus basalis of Meynert (nbM), caudally [9,18]. Degenerative changes in the cortical-projecting cholinergic neurons are known to contribute to the cognitive and attentional deficits seen in AD [19,20]. Several clinical pathological investigations report that similar pathology can develop chronically after TBI [21]. For example, the cerebral cortex of individuals who died as a consequence of head injury display reduced activity of the synthetic enzyme for Ach, choline acetyltransferase (ChAT), as well as morphological alterations and reduced ChAT immunoreactivity of the cholinergic nbM neurons [22]. TBI is also
associated with an acute down-regulation of the vesicular Ach transporter (VChT, the regulator of Ach neurotransmission) and acetylcholinesterase (AChE, the Ach-degrading enzyme) [23,24]. Moreover, several studies indicated that donepezil, an AChE inhibitor, improves cognitive function and attention after TBI [25], although this remains controversial [26–28].

The mechanism(s) driving cholinergic nbM neuronal dysfunction following CTE remain unknown. It is possible that these neurons undergo alterations of their survival protein, nerve growth factor [29,30], which is retrogradely transported from the cortex to the nbM cholinergic neurons through a complex interaction with its two cognate receptors, the high-affinity NGF-specific cell survival tyrosine kinase (trkA) and the putative cell death associated low-affinity pan neurotrophin (p75NTR) receptor [31,32]. In AD, these neurons also develop intracellular lesions that appear as globose p-tau-positive NFTs [13,33]. Tau is a microtubule-associated protein involved in normal cytoskeleton function [34,35] and tau containing NFT within nbM neurons co-occurs within the cholinergic cell markers ChAT and p75NTR early in the onset of AD [36]. Recently, a linear model for NFT evolution has been proposed and that can be observed by antibodies directed against p-tau epitopes marking early, intermediate and late stages of NFT development during the progression of AD [37–43]. For example, tau phosphorylation at Serine 422 (pS422) was identified as an early event using the pS422 antibody, whereas tau truncation at aspartate 421 by caspase 3 (Asp421) and detected with a Tau C3 antibody occurs later during NFT formation [37]. A recent report identified tau conformational changes such as exposure of an amino-terminal motif and oligomerization as a very early pathological modification in the AD [44–46] and CTE [44] brain. While the involvement of p-tau containing NFTs within nbM neurons has been identified as a pathological lesion in CTE [9,11,47], to the authors’ knowledge there have been no studies investigating the progression of NFT pathology within the cholinergic neurons within the nbM in the CTE brain. The present study used site-specific tau antibodies to characterize NFT evolution in the cholinergic nbM neurons in tissue obtained at autopsy from former athletes and veterans exposed to repetitive mild TBI who were diagnosed neuropathologically with CTE [6,9,11,48].

**Methods**

**Subjects**

A total of 18 brains were obtained from former male American professional and college football and hockey players and boxers, including two who were also military veterans (see Table 1). Institutional review board approval for brain donation was obtained through the Boston University Alzheimer’s Disease Center (BUADC), CTE programme and the Bedford VA hospital. Institutional Review Board approval for post-mortem clinical record review, interviews with family members and neuropathological analysis were obtained through Boston University School of Medicine.

**Clinical evaluation**

Clinical evaluation was performed according to the recent guidelines described by Mez et al. [49]. Briefly, a retrospective
clinical evaluation was performed to obtain subject demographics including repetitive head injury exposure, substance use, medical, social and family histories. This evaluation is made up of a combination of online surveys and telephone calls between research personnel and family members as well as close friends of the individual. The collection of data is accomplished using an unstructured interview with either a behavioural neurologist or neuropsychologist (for details see Mez et al. [49]). Researchers performing the tests are blinded to the pathological examinations.

Neuropathological evaluation

BUADC Brain Bank performed the neuropathological staging of CTE as previously described [9,11,50]. Briefly, paraffin-embedded sections were processed histologically with Luxol fast blue, haematoxylin and eosin and Bielschowsky’s silver staining and immunohistochemically using antibodies against phosphorylated tau (p-tau; AT8; Pierce Endogen, Rockford, IL; 1:2000), alpha-synuclein (rabbit polyclonal, Chemicon, Temecula, CA; 1:15 000), amyloid-β (Aβ; 6F/3D; Dako North America, Inc., Carpinteria, CA; 1:2000), TDP-43 (Abcam, Cambridge, MA; 1:1000), phosphorylated TDP-43 (pTDP-43; pS49/410 mouse monoclonal; Cosmo Bio Co, Tokyo, Japan; 1:2000) and the phosphorylated neurofilaments, SMI-31 and SMI-34 (1:000, BioLegend, San Diego, CA) as previously described [7]. Neuropathological diagnosis was made blinded to the individual’s clinical history and confirmed by two board certified neuropathologists. Following neuropathological staging, the paraffin blocks containing the nbM were shipped to the Barrow Neurological Institute (Phoenix, AZ), where additional sections were collected for use in the present study.

Immunohistochemistry of the basal forebrain

Sections from a total of 18 cases containing the nbM and an additional seven cases containing the vertical limb of the diagonal band of Broca (VDB) were immunohistochemically evaluated. However, only the cases containing nbM were used for quantitation and are included in Table 1. Sections were slide mounted, deparaffinized, rehydrated and boiled in a citric acid solution (pH 6) for 5 minutes. Sections were immuno stained with either a goat polyclonal antibody against choline acetyltransferase (ChAT; dilution 1:500; Millipore, Billerica, MA), a mouse monoclonal antibody against human p75NTR (1:500; Thermo Scientific, Waltham, MA), a rabbit polyclonal antibody against the phospho-tau epitope pS422 (1:500; Thermo Fisher Scientific Pierce, Rockford, IL) [42,51], a mouse monoclonal antibody against the tau Asp421 cleavage neoepitope, Tau C3 (1:50, dilution; Thermo Scientific) [35,37,52], a mouse monoclonal TOC1 (tau oligomeric complex 1) antibody (1:500; a gift from the late Dr Lester ‘Skip’ Binder) that is selective for non-fibrillar tau oligomers [53,54] and a mouse monoclonal antibody TNT1 antibody (1:3000; a gift from Dr Lester ‘Skip’ Binder) that recognizes the phosphatase activating domain (PAD) region of tau, which is involved in the inhibition of anterograde, kinesin-based fast axonal transport (FAT) by activating axonal protein phosphatase 1 (PP1) and glycogen synthase kinase 3 (GSK3) [55] and the rabbit polyclonal antibody against the autophagy marker p62 or SQSTM1 (1:500 dilution; Thermo Fisher Scientific Pierce, Rockford, IL) in a 0.1 M TBS/1% normal goat or horse serum solution overnight at 4°C. Additional sections were stained for Aβ and TDP-43 using the 6E10 monoclonal antibody (1:1000; Covance, Princeton, NJ) that recognizes both the Aβ precursor protein (APP) and Aβ [56,57] and the rabbit polyclonal anti TAR-DNA binding protein-43 (TDP-43) (1:5000; ProteinTech Group, Chicago, IL), respectively. Sections stained for TDP-43 and APP/Aβ were processed for antigen retrieval using Target Antigen Retrieval Solution, pH 9.0 (DAKO, Carpinteria, CA) for 20 minutes in a steamer [58] and 88% formic acid for 7 minutes, respectively. Sections were incubated in 0.1 M Tris-buffered saline (TBS, pH 7.4) containing 0.1 M sodium periodate to eliminate endogenous peroxidase activity, blocked with 0.1 M TBS containing 0.25% Triton X-100 and 10% normal goat serum (NGS; Vector Labs, Burlingame, CA) for 1 hour. Sections were then incubated with biotinylated goat anti-mouse IgG (1:500; Vector Labs, Burlingame, CA), goat or horse anti-rabbit (1:200, Vector Labs) and processed with avidin-biotin complex reagent (ABC; Vector Labs). Tissue was then developed in 0.05% 3', 3'-diaminobenzidine (DAB) and 0.005% H2O2, resulting in a brown reaction product. Slides were dehydrated in a series of graded concentrations of ethanol (70, 90, 95 and 100%), cleared in xylene and cover-slipped using DPX (Electron Microscopy sciences, Hatfield, PA). Select sections were counterstained with cresyl violet for cytoarchitectural analysis.

Dual immunohistochemistry

A second series of sections were dual-immunostained with either a rabbit (1:250; Millipore) or a mouse (1:500; Thermo Scientific) anti human p75NTR antibody, a well-established marker for human cholinergic basal forebrain (CBF) neurons [59–61] and each tau antibody (pS422, TOC1 or TNT1). Due to methodological difficulties, dual labeling was unavailable in tissue from each case using the rabbit polyclonal p75NTR antibody and the monoclonal TOC1 and TNT1 antibodies, making it difficult to accurately quantitate double stained neurons in these experiments. Therefore, the dual neuronal quantitation was performed only on sections concurrently stained with the mouse monoclonal p75NTR and pS422 antibodies. After visualizing p75NTR (as described above), tissue was incubated with an avidin/biotin blocking kit (Vector Laboratories) and any remaining peroxidase activity was quenched with 3% H2O2 for 30 minutes at room temperature. Blocking buffer was re-applied for 1 hour at room temperature and the tissue was incubated in the second primary antibody (pS422, TOC1 or TNT1) overnight at 4°C and next day for 1 hour at room temperature before incubation with the appropriate biotinylated secondary antibody (1:500 in dilution buffer, Vector Laboratories) for 2 hours at room temperature. Tissues were then incubated in ABC solution as described above. Sections were processed with the Vector SG Substrate Kit (blue reaction product, Vector Laboratories) according to the manufacturer’s protocol. This dual staining resulted in an easily identifiable two-coloured profile: brown for p75NTR and dark blue/black for pS422, TOC1 and TNT1 positive profiles. Slides were air-dried, dehydrated, cleared in xylene and cover-slipped.

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Tau pathology with cholinergic neurons in CTE 1401
Omission of primary antibodies resulted in no detectable immunostaining (data not shown). AD basal forebrain tissue was processed in parallel with CTE tissue as a positive control for each antibody and to compare the morphological characteristics of NFT pathology between the two disorders (see Figure 1).

Immunofluorescence and histofluorescence
To examine the fibrillar nature of NFTs within cholinergic neurons, sections were double stained for p75NTR and X-34, a highly fluorescent derivative of Congo Red, which detects the full spectrum of AD amyloid pathology with greater sensitivity than thioflavin S [62,63]. The pan-amyloid dye

Figure 1. Photomicrographs of cresyl violet stained sections delineating the cholinergic sub-fields (Ch4) examined within the nbM from rostral to caudal: anteromedial (Ch4am) (a), anterolateral (Ch4al) (b) and dorsal (Ch4id) and ventral (Ch4iv) intermediate (c); a1, b1 and c1, insets showing higher magnification images of Ch4am, Ch4al and Ch4id neurons from panels a, b and c, respectively. (d and e) Dual immunolabeled p75NTR/pS422 neurons (dark blue; arrows) and single labeled p75NTR (brown) neurons in the VDB (Ch2) in a CTE stage III (d) and IV (e) case, respectively. (f and g) Photomicrographs of ChAT (brown) and p75NTR-ir (brown) neurons containing pS422-ir inclusions (dark/light blue; arrows) within the nbM anteromedial subfield in CTE stage IV. (h and i). Images of the anteromedial sub-field of the nbM showing dual immunolabelled ChAT (brown)/pS422 positive neurons (dark blue; arrows) and single immunolabelled ChAT neurons (brown) (h) and double immunolabelled p75NTR/pS422 positive neurons (dark and light blue; arrows) and single-labelled p75NTR neurons (brown) (i) from an AD case, respectively. (j–m). Photomicrographs of TOC1 (j), TNT1 (k), Tau C3 (l) and p62 (m) positive neurons and neuropil threads in the anterior subfields of the nbM of an AD case. Note the extent of oligomer TOC1 and TNT1 positive threads compared to absence of neuropil thread Tau C3 and p62 immunostaining. Tissues were counterstained with cresyl violet in panels j–m. ac, anterior commissure; bv, blood vessel. Scale bars: 500 µm (a), 250 µm (b), 200 µm (c), 100 µm (b1, c1), 80 µm (a1), 75 µm (j, l, m), 60 µm (f), 50 µm (h, i, k), 40 µm (g) and 30 µm (d, e).
X-34 labels β-sheet structure of Aβ fibrils in plaques as well as tau fibrils in NFT, dystrophic neurites and neuropil threads [63]. Briefly, sections were deparaffinized, rehydrated and treated with Protease XXIV (Sigma, St. Louis, MO) for 5 minutes at 37°C and washed in potassium phosphate buffer (KPBS, pH 7.4). To reduce autofluorescence, sections were incubated in 0.25% KMnO₄ in KPBS for 20 minutes, rinsed in KPBS and incubated in 1% potassium meta-bisulphite and oxalic acid for 5 minutes at room temperature. Subsequently, slides were washed in Tris buffered saline (TBS, pH 7.4), followed by several rinses in TBS/0.25% Triton X-100 (TTBS) and incubated in a 3% goat serum/TBS Triton X-100 blocking solution for 1 hour at room. Tissue was then incubated with mouse anti-p75NTR (1:50 dilution; Thermo Scientific) in TTBS/1% goat serum overnight at 4°C, followed by goat anti-mouse AlexaFluor 594 secondary antibody (1:200; Jackson ImmunoResearch Lab, West Grove, PA) at room temperature for 1 hour. After several washes in TBS and KPBS, sections were then incubated in 100 µM X-34 [63] for 10 minutes at room temperature followed by a brief wash in tap water and incubation in 80% ethanol for 2 minutes at room temperature. Sections were then rinsed in running tap water for 10 minutes and cover-slipped with Fluoromount-G (Southern Biotech, Birmingham, AL). Stains were imaged using an Olympus BX53 microscope with an X-Cite 120Q fluorescence attachment. Immunofluorescence p75NTR profiles were detected using the TRITC filter and X-34 was detected using an ultraviolet filter set. Single p75NTR and X-34 images were merged using the Layer blend option in Photoshop 6.0.

Quantitation of NFT profiles within the nbM

Counts of double pS422/p75NTR and single p75NTR, TOC1, TNT1, Tau C3 and p62 positive neurons were performed within two sections representing the extent of the nbM in each case, with a Nikon Microphot-FXA microscope at 10x magnification. For each antibody, counts of all p-tau labelled neurons were averaged per case [57] and normalized to the total number of p75NTR positive neurons. Results are presented as a percentage of cell counts. Fiduciary landmarks were used to prevent repetitive counting of labelled profiles.

Table II. Progression of symptoms by CTE pathological stage.

<table>
<thead>
<tr>
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<th>II</th>
<th>III</th>
<th>IV</th>
<th>p-valuea</th>
<th>Pairwise comparison</th>
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<tbody>
<tr>
<td>Age Sport Begun (y)</td>
<td>8.57 ± 3.98*</td>
<td>11.80 ± 2.0</td>
<td>12.66 ± 2.8</td>
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<td>–</td>
</tr>
<tr>
<td>Years of Play</td>
<td>16.14 ± 4.9</td>
<td>18.60 ± 3.2</td>
<td>20.33 ± 6.1</td>
<td>ns</td>
<td>–</td>
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<tr>
<td>Age at Onset Symptoms (y)</td>
<td>33.57 ± 16.1</td>
<td>32.40 ± 6.3</td>
<td>50.33 ± 16.2</td>
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<td>–</td>
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<tr>
<td>Interval Between Retirement and Symptoms (y)</td>
<td>8.85 ± 14.2</td>
<td>2.00 ± 5.09</td>
<td>17.33 ± 15.2</td>
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<td>–</td>
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<tr>
<td>Interval Between Symptom Onset and Death (y)</td>
<td>8.28 ± 10.6</td>
<td>22.40 ± 13.6</td>
<td>24.66 ± 15.4</td>
<td>ns</td>
<td>–</td>
</tr>
<tr>
<td>Age at Death (y)</td>
<td>41.85 ± 19.2</td>
<td>54.80 ± 9.3</td>
<td>75.00 ± 6.8</td>
<td>0.002</td>
<td>II &lt; IV</td>
</tr>
</tbody>
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*mean ± SD; ns, no significance
*One-way ANOVA with Holm-Sidak post hoc

Statistical analysis

Demographic and clinical characteristics as well as cell counts were evaluated across pathological CTE stages II, III and IV using a one-way analysis of variance, Kruskal-Wallis test followed by Holm-Sidak and Dunn’s post-hoc test for multiple comparisons as appropriate and Spearman rank for correlations (Sigma Plot 12.5, Systat Software, San Jose, CA). Statistical significance was set at 0.05 (two-tailed).

Results

Case demographics

Table I shows the demographics of the 18 male cases with a history of repetitive mild TBI. Cases were categorized as CTE Stage II, III or IV according to the criteria published by McKee et al. [9,11]. Stage II cases (n = 7; mean age = 41.9 ± 19.2) included three National football players, one semi-professional American football player, one American college football player, one high school football/wrestler/pole vaulter and one professional hockey player. Stage III cases (n = 5; mean age = 54.8.9 ± 9.3) were all National football league football players. Stage IV cases (n = 6; mean age = 75.0 ± 6.8) included five National football league players, of which two were also military veterans, and a professional boxer. The groups differed by age (p = 0.002), with stage IV cases significantly older compared to stage II, but no differences were found when compared by age to the time at which sport begun, years of play, age at symptom onset, time interval between retirement and symptom onset and interval between symptom onset and death (Table 2).

Older age when sport begun was associated with older age at symptoms onset (r = 0.60, p < 0.008), longer interval between symptoms onset and death (r = 0.48, p < 0.04) and with older age at death (r = 0.72, p < 0.0003) (Table 3). Older age at symptoms onset was also associated with longer interval between symptoms onset and retirement from sport (r = 0.85, p < 0.0001) and with older age at death (r = 0.71, p = 0.0005). In addition, older age at death was associated with longer intervals from symptom onset to either retirement from sport (r = 0.52, p = 0.02) or death (r = 0.64, p = 0.003) (Table 3).
Pathological findings

Consistent with previous reports [13,64], other pathologies were present in the subjects with CTE: 13/18 (72%) had pTDP-43 inclusions; 6/18 (33%) had diffuse Aβ plaques; 3/18 (17%) had neuritic Aβ plaques; and 1/18 (6%) had Lewy body disease. Co-morbid pathologies were more common in older subjects with CTE with a higher CTE stage.

Tau pathology within the nbM and VDB

Nissl stained sections revealed clusters of large hyperchroomic magnocellular neurons within the nbM extending anteriorly at the level of the crossing of the anterior commissure to the emergence of the commissure at the level of the amygdala (Figures 1(a–c)). In seven cases, the VDB was included in the paraffin block; however, this area was not included in the primary analysis (Figures 1(d) and (e)). The phenotype of the cholinergic neurons was determined using either an antibody against ChAT or p75NTR, accepted markers for cholinergic neurons in the human basal forebrain [65]. In agreement with a previous report [22], this study was unable to consistently visualize ChAT positive neurons within the nbM (Figure 1(f)) across the cases examined, most likely due to antibody fixation interactions. Therefore, quantitative and qualitative analysis were performed using the p75NTR antibody (Figure 1(g)). For all cases, a section containing the nbM from an AD case was included as a positive control for each tau antibody (Figures 1(h–m)). pS422, TOC1, TNT1, Tau C3 and p62 positive neurons were globose in appearance, as seen in the nbM in AD [66]. Qualitative analysis revealed that there were more pS422-ir profiles (Figure 1(h and i)) compared to oligomeric TOC1 (Figure 1(j)), TNT1 (Figure 1(k)), Tau C3 (Figure 1(l)) and p62 (Figure 1(m)) profiles in the nbM, similar to AD [13].

In CTE cases, mainly in stages III and IV, many p75NTR positive neurons appeared globose in shape, indicative of tangle bearing neurons [13,67]; this was confirmed in adjacent sections immunostained with pS422 (Figures 2(a–i)), oligomeric TOC1 (Figures 2(j–n)), TNT1 (Figures 2(o–s)) and TauC3 (Figures 3(a–c)). Numbers of TOC1, TNT1, pS422, Tau C3 and p62 positive nbM neurons increased with more advanced CTE stage (Figures 2(a–g) and 3(a–f)). Dual p75NTR and pS422 immunostaining revealed co-labelled as well as single p75NTR and pS422 neurons within the nbM at each CTE stage (Figures 2(a–e)). At any CTE stage, the greatest number of tau positive neurons was observed using the pS422 antibody (Table IV, Figures 2(a–e)). In stages III and IV cholinergic neurons display granular pS422 staining suggestive of an early stage of tangle development (Figures 2(d) and (e)). Both p75NTR/pS422 (Figures 2(f and g)) and single pS422 (Figures 2(h and i)) containing neurons displayed twisted tau filamentous resembling skeins of yarn [68], indicative of cellular degeneration [33]. The same morphological changes in the nbM cholinergic neurons were observed using the other tau markers (Figures 2(m), (n), (r) and (s)). Likewise, the greatest extent of immunoreactive neurites in the nbM was seen in stage IV, using pS422 (Figure 2(c)) and TOC1 (Figure 2(l)) antibodies, followed by TNT1 (Figure 2(q)) and to a lesser degree Tau C3 (Figure 3(c)) and p62 (Figure 3(f)). This study also observed pS422, TOC1 and TNT1 positive astrocytes surrounding large blood vessels within the nbM, mainly in stages III and IV (Figures 3(g–i)). Similar NFT pathology was seen in the VDB in stage III and IV (Figures 1(d) and (e)).

Quantitative analysis revealed that more advanced CTE pathological stage was associated with increased numbers of neurons positive for all tau markers and for p62 within the nbM (Table IV and Figure 4). The percentage of double pS422/p75, and single pS422 and TOC1 and TNT1 positive nbM neurons was significantly different among the three CTE stages (p < 0.01 for all markers) with stage IV greater than stage II but not stage III (Table IV). The three CTE groups also differed by the number of Tau C3 (p = 0.009) and p62 (p = 0.002) positive neurons, with stages III and IV greater than stage II (Table 4). Approximately 50% of all pS422 neurons were p75NTR dual stained across all stages (Table 4).

Dual histofluorescence for fibrillar tau in the nbM

Double fluorescence analysis revealed that p75NTR immunoreactive nbM neurons contained NFT-like X-34 positive tau fibrils in stages III and IV and to a lesser extent in stage II (Figures 5(a–f)). In stages III and IV, most of the NFTs were single stained for X-34 (Figures 5(d–f)). X-34 positive plaques were not seen in the nbM at any CTE stage. However, in two of the 18 cases examined X-34 positive plaques were seen in the insular cortex.

Aβ immunostaining in the nbM

To determine the involvement of beta amyloid in the nbM in CTE, sections were immunostained using the 6E10 monoclonal antibody. Only diffuse, but not cored, Aβ plaques and vascular Aβ deposits were seen in five stage IV CTE cases (Figures 5(g–i)). Intracellular Aβ/APP immunoreactivity was not seen in nbM neurons in any of the CTE cases examined (Figures 5(g) and (h)). In contrast, both diffuse and cored/neuritic Aβ/APP plaques and vascular amyloid deposits were found in the insula and anterior temporal cortex in all stage IV CTE cases examined (Figures 5(i) and (j)).

TDP43 immunostaining in the nbM

To examine whether TDP-43 was involved in the pathology of nbM neurons, sections were immunostained using a TDP-43 antibody, which reveals intact and post-transcriptional changes in this primarily nuclear protein. In addition to the non-pathological nuclear TDP-43 immunoreactive -ir) in CTE nbM neurons, lenticular shaped TDP-43-ir inclusions (Figures 5(k) and (l)) were observed within nbM neurons in CTE, but not in the AD basal forebrain (Figure 5(m)).

Correlations between percentage of NFTs in the nbM and demographics

Across all CTE stages, there was a strong statistically significant association among the pS422/p75NTR, pS422, TOC1, TNT1, Tau C3 and p62 positive neurons (Table 5). A greater percentage of pS422/p75NTR, pS422 and TNT1
labelled neurons were significantly correlated with age at symptom onset (Figures 6(a–c)) (pS422/p75NTR: r = 0.053, p = 0.02; pS422: r = 0.049, p = 0.03; TNT1, r = 0.50, p = 0.04), interval between symptom onset and death (pS422/p75NTR: r = 0.061, p = 0.006; pS422: r = 0.62, p = 0.005; TNT1, r = 0.54, p = 0.02, respectively) and age at death (Figures 6(d–f)) (pS422/p75NTR: r = 0.075, p = 0.000 07; pS422: r = 0.73, p = 0.0003; TNT1, r = 0.67, p = 0.002) (Table 6). A greater percentage of TOC1, TauC3 and p62-ir nbM neurons were significantly associated with longer intervals between symptom onset (TOC1: r = 0.62, p = 0.05; Tau C3: r = 0.67, p = 0.002; p62: r = 0.68, p = 0.002) and older age at death (TOC1: r = 0.65, p = 0.003; Tau C3: r = 0.65, p = 0.004; p62: r = 0.51, p = 0.03) (Table 6).

Discussion

The present findings demonstrate that cholinergic nbM neurons develop progressive NFT pathology in CTE similar to the changes with p-tau-positive neurofibrillary pathology seen in prodromal and frank AD [17]. These findings support the concept that cortical projecting
neurons belonging to cholinergic and likely other neurotransmitter systems (i.e. locus coeruleus noradrenergic neurons [69]) are vulnerable following CTE. Moreover, cholinergic nbM neurons in CTE develop NFT within nbM neurons in a sequence characterized by the appearance of the pre-tangle marker phospho-tau epitope pS422, oligomeric tau (TOC1) and Tau C3, a late stage apoptotic marker, similar to that found in AD [66]. These results confirm previous reports of NFTs within the nbM [9] and support findings showing these specific forms of p-tau pathology in the frontal cortex of individuals with CTE [44]. This study also reports that the nbM fronto-cortical neurons [44] are immunoreactive to TNT1, another pre-tangle antibody that recognizes PAD region of tau, involved in the inhibition of anterograde, kinesin-based FAT by activating axonal PP1 and GSK3 [55]. Significantly greater numbers of TOC1, TNT1 and pS422 positive neurons in stage IV were observed compared to stage II but not stage III, which may be due to the single stage III case that displayed NFT counts similar to that seen in stage IV. There was also a significantly greater number of Tau C3 and p62 positive neurons in stages III and IV compared to stage II. The expression of p62 positive neurons across all pathological stages of CTE suggests that cholinergic nbM neurons undergo autophagic as well as cytoskeletal alterations in CTE. The display of p62 suggests that these neurons are experiencing problems related to clearance of cellular debris due to a breakdown of autophagic mechanism similar to that seen in AD [70–72]. Across CTE stages, dual labeled p75NTR/pS422 but also single p75NTR and pS422 labeled neurons were observed within the nbM. Compared to stage II, cases classified as stage IV had significantly more p75NTR neurons co-labelled with pS422. These finding indicate that nbM neurons develop NFTs gradually across the pathological stages of CTE. It remains to be determined whether NFT changes are present in nbM neurons at the very onset of pathological CTE (e.g. stage I). Regardless, the present data indicate that development of pre-tangle changes in the cholinergic nbM neurons may contribute to cholinergic dysfunction [66] and play a role in the onset of cognitive impairment in CTE [21,73]. The protracted development of NFTs within the cholinergic neurons of the nbM may coincide with the pre-symptomatic period

Figure 3. Photomicrographs of Tau C3 (a–c) and p62 (d and f) immunolabelling showing many more globose Tau C3 and p62-positive neurons in the anterior sub-fields of the nbM in a CTE stage IV than stage II. Note the lack of nbM Tau C3 positive neurons in stage II. Photomicrographs of the double immunolabelling for pS422/p75NTR (g), TOC1/p75NTR (h) and single immunolabelling for TNT1 (i) showing pS422 (blue), TOC1 (blue) and TNT1 (brown) immunostained astrocytes near to a blood vessel in anteromedial sub-field of the nbM in a CTE stage IV case. Arrow indicates a p75NTR positive cholinergic neuron. bv, blood vessel. Tissues in panels a-c and e were counterstained with cresyl violet. Scale bars: 90 µm (a–d), 70 µm (e, f), 40 µm (g) and 25 µm (h, i).
between the exposure to head trauma and the onset of clinical symptoms in CTE and may mark a window of opportunity for therapies.

The present finding of NFT pathology progression within cholinergic nbM neurons may underlie the observation that ChAT activity in the inferior temporal [74], cingulate and parietal cortex [22], each of which are innervated by select cholinergic sub-fields of the nbM is significantly reduced in TBI [18]. Despite the loss of cholinergic activity in the cortex of CTE, post-synaptic cholinergic nbM neurons may underlie the observation that Ach at the synapse have been used to treat mild-to-moderate AD [76–81] and several of these drugs have been identified as a treatment option for patients with TBI [4,21]. Donepezil, a centrally acting AChE inhibitor, has been shown to improve cognitive function and attention in TBI patients [25,82,83]. On the other hand, several studies reported no significant change in cognitive function after Donepezil treatment of TBI [26–28]. Interestingly, a study suggested that Donepezil administration may be beneficial early following TBI [27]. Since formation of NFTs within the nbM is an early event and continues progressively during the pathological course of CTE, therapies preventing the build-up of the past several years a group of drugs targeting the re-uptake of Ach at the synapse have been used to treat mild-to-moderate AD [76–81] and several of these drugs have been identified as a treatment option for patients with TBI [4,21]. Donepezil, a centrally acting AChE inhibitor, has been shown to improve cognitive function and attention in TBI patients [25,82,83]. On the other hand, several studies reported no significant change in cognitive function after Donepezil treatment of TBI [26–28]. Interestingly, a study suggested that Donepezil administration may be beneficial early following TBI [27]. Since formation of NFTs within the nbM is an early event and continues progressively during the pathological course of CTE, therapies preventing the build-up of

### Table III. Correlation between symptoms and death by CTE pathological stage.

<table>
<thead>
<tr>
<th>Years of Play</th>
<th>Age at Onset Symptoms (y)</th>
<th>Interval between retirement and symptoms (y)</th>
<th>Interval between symptom onset and death (y)</th>
<th>Age at Death (y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at Sport</td>
<td>–</td>
<td>r = 0.60</td>
<td>ns</td>
<td>r = 0.48</td>
</tr>
<tr>
<td>Begun</td>
<td>–</td>
<td>p = 0.008</td>
<td>ns</td>
<td>p = 0.04</td>
</tr>
<tr>
<td>Years of Play</td>
<td>–</td>
<td>–</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Age at Onset</td>
<td>–</td>
<td>r = 0.85</td>
<td>ns</td>
<td>r = 0.71</td>
</tr>
<tr>
<td>Symptoms (y)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ns</td>
</tr>
<tr>
<td>Interval</td>
<td>–</td>
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<td>between</td>
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<tr>
<td>retirement</td>
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<td>and symptoms</td>
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<td>(y)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</tbody>
</table>

*ns, no significance

### Table IV. Percent of double pS422/p75 and single pS422, TOC1, TNT1, Tau C3 and p62 NFTs within the nbM by CTE pathological stage.

<table>
<thead>
<tr>
<th></th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>p-value</th>
<th>pairwise comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>pS422/p75</td>
<td>3.19 ± 3.30*</td>
<td>11.16 ± 7.4</td>
<td>24.68 ± 6.9</td>
<td>0.002*</td>
<td>II&gt;IV</td>
</tr>
<tr>
<td>pS422</td>
<td>6.18 ± 5.2</td>
<td>26.65 ± 15.4</td>
<td>48.90 ± 23.6</td>
<td>0.001*</td>
<td>II&gt;IV</td>
</tr>
<tr>
<td>TOC1</td>
<td>3.62 ± 2.9</td>
<td>14.13 ± 5.6</td>
<td>28.95 ± 24.3</td>
<td>0.001*</td>
<td>II&gt;IV</td>
</tr>
<tr>
<td>TNT1</td>
<td>2.03 ± 2.8</td>
<td>10.01 ± 8.3</td>
<td>17.62 ± 10.5</td>
<td>0.008b</td>
<td>II&gt;IV</td>
</tr>
<tr>
<td>Tau C3</td>
<td>1.80 ± 3.1</td>
<td>13.00 ± 5.9</td>
<td>15.64 ± 11.8</td>
<td>0.009b</td>
<td>II&lt;III, IV</td>
</tr>
<tr>
<td>p62</td>
<td>0.36 ± 0.4</td>
<td>3.10 ± 3.3</td>
<td>8.69 ± 4.4</td>
<td>0.002b</td>
<td>II&lt;III, IV</td>
</tr>
</tbody>
</table>

*mean ± SD

*Kruskal-Wallis with Dunn’s post hoc

bOne-way ANOVA with Holm-Sidak post hoc

### Table V. Correlation between the percentage of tau and autophage NFTs within the nbM by CTE pathological stage.

<table>
<thead>
<tr>
<th></th>
<th>pS422/75NTR</th>
<th>pS422</th>
<th>TOC1</th>
<th>TNT1</th>
<th>Tau C3</th>
<th>p62</th>
</tr>
</thead>
<tbody>
<tr>
<td>r</td>
<td>p = 0.96 p&lt; 1*10⁻⁶</td>
<td>r = 0.93 p&lt; 1*10⁻⁶</td>
<td>r = 0.91 p&lt; 1*10⁻⁶</td>
<td>r = 0.86 p&lt; 1*10⁻⁶</td>
<td>r = 0.81 p&lt; 1*10⁻⁶</td>
<td></td>
</tr>
<tr>
<td>pS422</td>
<td>–</td>
<td>r = 0.97 p&lt; 1*10⁻⁶</td>
<td>r = 0.91 p&lt; 1*10⁻⁶</td>
<td>r = 0.86 p&lt; 1*10⁻⁶</td>
<td>r = 0.80 p&lt; 1*10⁻⁶</td>
<td></td>
</tr>
<tr>
<td>TOC1</td>
<td>–</td>
<td>–</td>
<td>r = 0.90 p&lt; 1*10⁻⁶</td>
<td>r = 0.88 p&lt; 1*10⁻⁶</td>
<td>r = 0.81 p&lt; 1*10⁻⁶</td>
<td></td>
</tr>
<tr>
<td>TNT1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>r = 0.75 p&lt;1*10⁻⁴</td>
<td>r = 0.67</td>
<td></td>
</tr>
<tr>
<td>Tau C3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>p = 0.002</td>
</tr>
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</table>
p-tau within cholinergic nbM cortical and hippocampal projection neurons could represent a therapeutic target for CTE, similar to AD.

A proportion of subjects with CTE displayed both tau pathology and Aβ plaques and a sub-set also meet the pathological criteria for AD [84]. In a study of a heterogeneous cohort of deceased CTE cases diffuse or neuritic amyloid plaques were seen in the cortex in just over half of the subjects [84], a finding also confirmed in the present report. However, it was found that even at advanced stages of CTE (stage IV) with neocortical neuritic Aβ plaques, the nbM contains only diffuse plaques. In AD, it has been suggested that cortical plaque pathology can initiate a retrograde cellular response within these cholinergic neurons [85] that triggers

Figure 5. (a–f) Fluorescence images of single p75<sub>NTR</sub> (red) (a and d) and X-34 (green) (b and e) staining and merged-images (c and f) showing limited X-34 positive neurons (b) and the absence of double p75<sub>NTR</sub>/X-34 positive neurons (c) in the nbM in a CTE stage II case, while many more fibrillary X-34 positive neurons (e and f) were seen in the anterior sub-fields of the nbM in a CTE stage IV case. Arrow in (f) indicates a double immunolabelled p75<sub>NTR</sub>/X-34 neuron (yellow). (g) Low magnification image of scattered APP/Aβ (6E10) labelled deposits (brown; arrows) in the nbM anteromedial sub-field of a CTE stage IV case. (h) High power image of APP/Aβ-positive blood vessel (arrows) from the area boxed in panel g; note the lack of intra-neuronal APP/Aβ immunostaining in the large hyperchromic nbM neurons (blue). (i and j) Images showing APP/Aβ positive deposits in blood vessels (bv) in the insular cortex as well as APP/Aβ reactive cored neuritic plaques in the temporal cortex (j) from a stage IV CTE case. (k–m) Images showing nuclear TDP-43 staining (brown) in the nbM neurons from a CTE stage II (k), IV (l) and an AD case (m). Note the presence of lenticular shaped TPD-43-ir inclusions (red arrows) within nbM neurons in a CTE stage II (k) and IV (l), but not in the AD case (m). Black arrows indicate NFT-containing neurons (light blue) in CTE and AD cases. Sections in panels g–j were counterstained with cresyl violet and sections in panels k–m were counterstained with hematoxylin. Scale bars: 200 µm (g), 100 µm (a–c), 50 µm (d–f, h, j), 40 µm (i) and 25 µm (k–m).
tau over-expression resulting in neuronal degeneration and cholinergic dysfunction. Whether a similar cortical process plays a role in NFT formation in cholinergic nbM neurons in subjects with CTE remains to be explored.

This study found that the percentages of pS422, TOC1, TNT1, Tau C3 and p62-ir nbM neurons are highly correlated across all pathological stages of CTE. These findings complement a biochemical study showing changes in soluble and insoluble tau fractions within the frontal cortex of CTE cases [44], suggesting that pS422, PAD exposure and tau oligomerization occur across brain regions. Interestingly, a correlation between the biochemical levels of Tau C3 was not found in the frontal cortex, suggesting that there may be some tau changes that are regionally specific in CTE [44] or that the analysis of total brain tissue for biochemistry include non-neuronal profiles (i.e. p-tau positive glia). These findings are also similar to the sequence of tau biochemical [66] and intraneuronal cytoskeletal changes seen in AD [33]. Here, pS422, TNT1 and TOC1 astrocytic pathology was observed in the nbM, suggesting that the tau pathology in both neurons and glial undergo similar modifications in this region. In fact, glia positive for these tau markers were shown within astrocytes, but not microglia, in CTE frontal cortex [44]. Importantly, pS422 containing tangles has been shown to be the strongest correlate of cognitive impairment in AD [66]. Whether a similar association exists in CTE remains to be investigated, although CTE stage correlates with the progression of clinical symptoms [9].

We observed that greater numbers of pre-tangle neurons positive for pS422 and TNT1 (a marker of neuronal anterograde axonal transport defect) as well as for TOC1 and Tau C3 were associated significantly with older age at symptom onset and age at death in each CTE stage. Stage II, red circles; Stage III, green circles; Stage IV, blue triangles.
onset, longer interval between symptom onset and death and older age at death, suggesting their potential biomarker value for the pathological progression of CTE. The findings suggest that the pathobiological evolution of p-tau positive NFTs within the nbM as well as in the frontal cortex [44] may contribute to cognitive symptoms in CTE. Diffuse axonal damage due to acceleration/deceleration head injuries results in axonal tearing or at a minimum disruption in axonal transport and it is possible that axonal damage in CTE involves impaired axonal transport leading to p-tau accumulation and NFT formation [86], as suggested in AD [17]. Abnormal TDP-43 nuclear inclusions were found in nbM neurons during the pathological progression of CTE, supporting earlier reports of these inclusions in cortical neurons in TBI/CTE cases [87]. Whether the TDP-43 inclusions seen within the nbM neurons effect gene transcription, RNA transcription, preRNA splicing or mRNA biogenesis in these neurons remains unknown.

There are several limitations to the findings presented in this study. This study examined a limited autopsy cohort containing a heterogeneous population of subjects with a history of CTE. Therefore, the cases examined here may not be representative of the larger CTE population. In addition, clinical histories were obtained retrospectively from family members and, thus, they may be subject to bias. Future prospective clinical and pathological investigations are needed to determine the interaction between cholinergic and other pathologies with cognitive impairment in subjects with CTE.

In summary, the present study provides evidence that the cholinergic nbM neurons are affected with NFT pathology early during the pathological stages of CTE. This progression of NFT pathology follows a linear model, which can be observed by antibodies directed against p-tau epitopes marking early, intermediate and late stages of NFT development similar to that seen during the progression of AD [37–42]. It was also found in the nbM of advanced CTE cases that the co-existence of Aβ pathology, when present, was restricted to diffuse and vascular Aβ deposits. Although neuritic Aβ plaques were absent from the nbM in all CTE cases examined, this type of Aβ pathology was seen in adjacent cortex. TDP-43 abnormal inclusions were also seen early in the pathological staging of CTE. The involvement of these lesions in the onset of dementia in CTE remains to be determined. Further studies are needed to determine the mechanisms driving the formation of NFTs and TDP-43 inclusions within the nbM and their interaction with other alterations that are also seen in AD [67].

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Declaration of interest
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