Gene Profiling of Nucleus Basalis Tau Containing Neurons in Chronic Traumatic Encephalopathy: A Chronic Effects of Neurotrauma Consortium Study

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Abstract

Military personnel and athletes exposed to traumatic brain injury may develop chronic traumatic encephalopathy (CTE). Brain pathology in CTE includes intracellular accumulation of abnormally phosphorylated tau proteins (p-tau), the main constituent of neurofibrillary tangles (NFTs). Recently, we found that cholinergic basal forebrain (CBF) neurons within the nucleus basalis of Meynert (nbM), which provide the major cholinergic innervation to the cortex, display an increased number of NFTs across the pathological stages of CTE. However, molecular mechanisms underlying nbM neurodegeneration in the context of CTE pathology remain unknown. Here, we assessed the genetic signature of nbM neurons containing the p-tau pretangle marker pS422 from CTE subjects who came to autopsy and received a neuropathological CTE staging assessment (Stages II, III, and IV) using laser capture microdissection and custom-designed microarray analysis. Quantitative analysis revealed dysregulation of key genes in several gene ontology groups between CTE stages. Specifically, downregulation of the nicotinic cholinergic receptor subunit β -2 gene (CHRNA2), monoaminergic enzymes catechol-O-methyltransferase (COMT) and dopa decarboxylase (DDC), chloride channels CLCN4 and CLCN5, scaffolding protein caveolin 1 (CAV1), cortical development/cytoskeleton element lissencephaly 1 (LIS1), and intracellular signaling cascade member adenylate cyclase 3 (ADCY3) was observed in pS422-immunoreactive nbM neurons in CTE patients. By contrast, upregulation of calpain 2 (CAPN2) and microtubule-associated protein 2 (MAP2) transcript levels was found in Stage IV CTE patients. These single-population data in vulnerable neurons indicate alterations in gene expression associated with neurotransmission, signal transduction, the cytoskeleton, cell survival/death signaling, and microtubule dynamics, suggesting novel molecular pathways to target for drug discovery in CTE.

Keywords: basal forebrain; brain trauma; CTE; expression profiling; genes; TBI

Introduction

CHRONIC TRAUMATIC ENCEPHALOPATHY (CTE), previously termed “dementia pugilistica,” is a neurodegenerative dementia associated with repetitive mild brain trauma. CTE has been identified in American football, ice hockey, rugby, and soccer players; boxers; wrestlers; and military personnel. This concussive dementia is pathologically characterized by the widespread distribution of neurofibrillary tangles (NFT) containing the hyperphosphorylated tau protein (p-tau), a hallmark of Alzheimer’s disease (AD), in the neocortex, limbic cortex, and subcortical regions,^{1–3} including cholinergic basal forebrain (CBF) neurons, located within

the nucleus basalis of Meynert (nbM).^{3–5} These CBF neurons form a continuum of perikarya extending from the septal diagonal band (Ch1–2) caudally throughout the nbM (Ch4),⁵ are associated with memory and attention dysfunction,⁶ provide the major source of acetylcholine (ACh) to the cortical mantle,⁵ and degenerate in AD and traumatic brain injury (TBI).^{4,7–10} There is a reduction in both the number of choline acetyltransferase (ChAT) positive neurons within the nbM as well as ChAT activity, following TBI.¹¹ In addition, there is also an acute downregulation of the regulator of ACh neurotransmission, the vesicular ACh transporter (vAChT) and acetylcholinesterase (AChE) after TBI.¹² Moreover, several studies indicate that the centrally acting United States Food and Drug Administration

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(FDA)-approved AChE inhibitor, donepezil, improves cognitive function as well as attention after TBI,¹³ although this remains controversial.^{14–16} Taken together, these findings suggest that the CBF cortical projection system is damaged after head trauma in humans, and may be impacted significantly in CTE.

Numerous studies have shown that NFT pathology is the best correlate of cognitive decline to date in AD.¹⁷ In this regard, we have shown that pretangle p-tau pathology in CBF neurons correlates with cognitive decline during the progression of AD.¹⁸ Recently, we reported that nbM neurons progressively develop intracellular tau-immunoreactivity across the CTE pathological stages proposed by McKee and colleagues,¹ similar to AD.^{3,4,18} In particular, there was a significant increase in pretangle phosphorylated pS422 and oligomeric tau proteins within these vulnerable neurons.⁴ Moreover, the numbers of neurons containing pretangles and tau oligomers correlated with age at symptom onset and age of death in CTE.⁴ These findings indicate that early tau changes during NFT development contribute to CBF degeneration in CTE. Moreover, it is likely that following an initial brain injury, there are delayed secondary alterations that develop over days, weeks, months, and possibly years, which result in the perturbation of normal homeostatic mechanisms that can activate cell death and impede pro-survival molecular cascades in CBF neurons with the nbM. In this regard, it has been suggested that TBI causes significant gene expression level changes within vulnerable populations of neurons.^{19,20} Understanding the molecular cellular pathophysiology underlying CTE injury should be considered key to the identification of putative new targets for the treatment of this disorder. However, our current understanding of the molecular mechanisms underlying neurodegeneration post-CTE in the human brain is limited. In this regard, the molecular fingerprint of CBF neurons within the nbM containing p-tau following TBI requires a strategy that will uncover expression alterations at the cellular level.

Although genomic approaches to study the molecular pathogenesis of brain trauma have been applied to homogenized cortical brain tissue from pre-clinical rodent TBI models,^{20–22} their application to the human condition is under-represented in the literature.²³ Over the past several years, we have employed single cell gene expression profiling to evaluate the molecular pathogenesis of human hippocampal and nbM neurons during the onset of AD.²⁴ Evidence from these gene expression studies reveal dysregulation of select classes of genes associated with neurotrophins and their receptors, cholinergic activity, synaptic proteins, and endosomal/lysosomal markers during the progression of AD.^{25–28} However, whether similar or different gene classes are altered in tangle-bearing nbM neurons in patients with CTE remains unknown. Herein, we assessed, for the first time, the genetic signature of nbM neurons containing the p-tau pretangle marker, pS422, obtained from subjects who came to autopsy and received a neuropathological staging assessment for CTE (Stages II, III, and IV) using a combination of laser capture microdissection (LCM) and custom-designed microarray analysis.^{29,30}

Methods

Subjects

A total of 17 brains obtained from male athletes who played American football and ice hockey as well as from boxers with a history of repetitive mild TBI that we used in a previous study (see Table 1).⁴ Two of these subjects were also military veterans. Institutional approval for brain donation was obtained through the Boston University School of Medicine. Institutional approval for postmortem clinical record review, interviews with family members, and neuropathological analysis was obtained through Boston University School of Medicine.

TABLE 1. DEMOGRAPHICS

Stage	Gender	Race	Sport/act	Age sport began (y)	Years of play	Age at onset of symptoms (y)	Interval between retirement and symptoms (y)	Interval between symptom onset and death (y)	Age at death (decade)
II	M	C	Football	16	15	65	34	5	7
	M	AA	Football	11	10	45	24	8	5
	M	C	Football	9	20	28	–1	2	3
	M	C	Semi-pro. football	8	18	30	4	32	6
	M	C	College football	6	15	21	0	4	2
	M	C	HS football, Wrestling, Pole vaulting	6	11	20	2.5	5	2
Mean ± SD				9.3 ± 3.7	14.8 ± 3.8	34.8 ± 17.2	10.5 ± 14.7	9.3 ± 11.2	4.1 ± 2.1
III	M	AA	Football	10	17	28	1	28	5
	M	C	Football	11	23	35	1	11	4
	M	C	Football	14	17	35	4	32	6
	M	C	Football	10	21	40	9	5	4
	M	C	Football	14	15	24	–5	36	6
Mean ± SD				11.8 ± 2.0	18.6 ± 3.2	32.4 ± 6.3	2.0 ± 5.0	22.4 ± 13.6	5.0 ± 1.0
IV	M	AA	Boxing	12	19	30	–1	43	7
	M	C	Football	10	30	57	17	22	7
	M	C	Football	16	13	68	39	11	7
	M	AA	Football	9	22	50	19	12	6
	M	AA	Football/Veteran	14	23	65	28	15	8
	M	C	Football/Veteran	15	15	32	2	45	7
Mean ± SD				12.6 ± 2.8	20.3 ± 6.1	50.3 ± 16.2	17.3 ± 15.2	24.6 ± 15.4	7.0 ± 0.6

AA, African American; C, Caucasian; HS, high school; M, male; Semi-pro, semiprofessional.

Clinical evaluation

Cognitive and behavioral changes and clinical status were determined through postmortem interviews with next of kin performed by a neuropsychologist blinded to postmortem neuropathological status.³¹ To this end, a semistructured telephonic interview was conducted regarding demographics, athletic history, military service, concussion and brain trauma history, medical history (including neurological, psychiatric, and substance use history), family history, social/occupational history, and reported/observed changes in mood, behavior, motor function, cognition, and activities of daily living.³¹ To evaluate cognitive, mood, and functional changes, modifications of standard measures/interviews were administered to informants to evaluate their perception of the subject in the months or years prior to death. A review of each individual's medical records was performed, and cases with other comorbidities were excluded.

Neuropathological evaluation

Paraffin-embedded sections were processed for neuropathological evaluation according to the procedure of the Veterans Affairs-Boston University-Concussion Legacy Foundation (VA-BU-CLF) Bain Bank as previously described.³² The following stains were applied to the tissue: Luxol fast blue, hematoxylin and eosin, Bielschowsky's silver, p-tau (AT8), α -synuclein, amyloid- β (A β), TAR DNA-binding protein (TDP)-43, phosphorylated TDP-43 (pTDP-43), SMI-31 and SMI-34.³³ Neuropathological diagnoses were made blinded by a board-certified neuropathologist.

CTE pathological staging

CTE classification was based on the four pathological stages described previously.³² In Stage I, there are isolated perivascular foci of p-tau NFTs, neuropil threads, and astrocytic tangles mostly in the depths of cerebral sulci of the superior, dorsolateral, lateral, and inferior frontal cortices as well as p-tau NFT degeneration in the locus coeruleus, amygdala, entorhinal cortex, hippocampus, medulla, and cingulate gyrus. In Stage II multiple foci of tau pathology at the depths of the sulci mostly in the superior, dorsolateral, lateral, and inferior frontal, anterior inferior and lateral temporal, inferior and superior parietal, insular and septal cortices, and substantia innominata were described. Stage III exhibits NFTs diffusely in the frontal, temporal and parietal cortices, mainly around small vessels and within the depths of sulci. The hippocampus, entorhinal cortex, amygdala, nbM, and locus coeruleus show extensive neurofibrillary pathology as well as frequent NFTs in the hypothalamus, substantia nigra, and dorsal and median raphe nuclei. Stage IV shows a significant loss in brain weight and pronounced frontal and temporal lobe atrophy. At the light microscopic level, there is severe spongiosis of cortical layer II and widespread neuronal loss, including the substantia nigra, patchy widespread myelin loss, extensive glial tangles, and small NFTs in a patchy irregular distribution throughout frontal, temporal, and parietal cortices as well as extensive NFT degeneration in the insula, septa, temporal cortex, amygdala, hippocampus, entorhinal cortex, substantia nigra, and locus coeruleus. In Stage IV, p-tau pathology also involves the cerebellum, medial lemniscus, and inferior olives of the medulla. CTE stage correlates with the progression of clinical symptoms.³²

Immunohistochemistry

An initial set of paraffin-embedded blocks containing the nbM was cut at 8 μ m, slide mounted, deparaffinized, rehydrated and boiled in a citric acid solution (pH 6) for 5 min, for antigen retrieval.⁴ After several washes in 0.1M Tris-buffered saline (TBS, pH 7.4), sections were incubated with 3% H₂O₂ for 30 min at room temperature to eliminate endogenous peroxidase activity, and blocked with 0.1 M TBS containing 0.04% Triton X-100 and 10%

normal goat serum (NGS; Vector Labs, Burlingame, CA) for 1 h. Subsequently, sections were incubated in a solution containing polyclonal rabbit antibody directed against the p-tau epitope pS422 (1:500; Thermo Fisher Scientific Pierce, Rockford, IL), a marker of pretangles mixed in a 0.1 M TBS/2% normal goat serum solution overnight at room temperature.^{18,34} The next day, sections were washed in TBS and then incubated with the secondary biotinylated goat anti-rabbit IgG (1:200; Vector Labs, Burlingame, CA), and processed with avidin-biotin complex reagent (ABC; Vector Labs, Burlingame, CA). Tissue was then developed in 0.05% 3', 3'-diaminobenzidine (DAB) and 0.005% H₂O₂ resulting in a brown reaction product. These sections were counter-stained with Cresyl Violet for identification of the nbM neurons. A second set of sections was dual labeled using the abovementioned pS422 tau antibody and a monoclonal antibody directed against p75^{NTR} (1:500; Thermo Scientific, Waltham, MA), an excellent marker of cholinergic nbM neurons in humans to identify non-tangle-bearing cholinergic neurons at the light microscopic level.^{4,35}

Dual-stained neurons were not evaluated for expression level changes, because of difficulties in consistently identifying these cells with the LCM. All slides were dehydrated in a series of graded concentrations of ethanol, (70, 90, 95, and 100%) but not cover-slipped.

Quantitation of the number of pS422 compared with p75^{NTR} neurons in the nbM

Counts of single labeled pS422 nbM neurons were performed within at least two sections using the Nikon Microphot-FXA microscope at 10 $\times$ magnification and normalized to the total number of p75^{NTR} positive neurons as previously described.⁴ Results are presented as percentage of cell counts.

Single-cell gene expression analysis

Single immunolabeled pS422 and p75^{NTR} nbM neurons (a total of 30 neurons per CTE case pooled/per assay) from CTE Stages II ($n=6$), III ($n=5$), and IV cases ($n=6$) were microaspirated by laser capture microdissection (PALM MicroBeam C IP, Carl Zeiss MicroImaging Inc., Thornwood, NY). The amplification of mRNA was performed with terminal continuation (TC) RNA amplification, which preserves the original quantitative relationships among the transcripts.^{36–38}

Microdissected nbM cells were homogenized in Trizol solution (ThermoFisher Scientific, Waltham, MA) and RNAs were reverse transcribed in the presence of the poly d (T) primer (100 ng/mL) and TC primer (100 ng/mL) in 1 $\times$ first strand buffer (ThermoFisher Scientific), 500 μ M deoxyribonucleotide triphosphate (dNTP)s, 5 mM dithiothreitol (DTT), 20 U of SuperRNase Inhibitor (ThermoFisher Scientific), and 200 U of reverse transcriptase (ThermoFisher Scientific). Single-stranded complementary DNAs (cDNAs) were digested with RNase H and re-annealed with the primers in a thermal cycler: RNase H digestion step at 37°C, 30 min; denaturation step at 95°C, 3 min; and primer reannealing step at 60°C, 5 min. This step generated cDNAs with double-stranded regions at the primer interface. Samples were then purified by column filtration (Montage PCR filters; Millipore, Billerica, MA). RNAs hybridization probes were synthesized by *in vitro* transcription with the use of ³³P incorporation in 40 mmol/L Tris (pH 7.5); 6 mmol/L MgCl₂; 10 mmol/L NaCl; 2 mmol/L spermidine; 2.5 mmol/L DTT; 125 μ mol/L adenosine triphosphate (ATP), guanosine triphosphate (GTP), and cytidine triphosphate (CTP); 2.5 μ mol/L cold uridine triphosphate (UTP); 20 U of RNase inhibitor; 2 kU of T7 RNA polymerase (Epicentre, Madison, WI); and 60 μ Ci of 33P-UTP (PerkinElmer, Waltham, MA). The labeling reaction was performed at 37°C for 4 h. Radiolabeled terminal continuation RNA probes were hybridized to custom-designed microarrays without further purification. Single-cell gene array expression was run in triplicate for each case.

Custom-designed microarray platforms and data analysis

Platforms consist of 1 μ g of linearized cDNA purified from plasmid preparations adhered to high-density nitrocellulose (Hybond XL, GE Healthcare, Piscataway, NJ).^{26,38,39} cDNAs were verified by sequence analysis and restriction digestion. Approximately 576 cDNAs were used on our custom array platform. Arrays were hybridized for 24 h in a solution consisting of 6 $\times$ saline-sodium phosphate-ethylenediaminetetraacetic acid, 5 $\times$ Denhardt's solution, 50% formamide, 0.1% sodium dodecyl sulfate (SDS), and denatured salmon sperm DNA (200 μ g/mL) at 42°C in a rotisserie oven. Following the hybridization protocol, arrays were washed sequentially in 2 $\times$ saline sodium citrate (SSC)/0.1% SDS, 1 $\times$ SSC/0.1% SDS, and 0.5 $\times$ SSC/0.1% SDS for 15 min each at 37°C. Arrays were placed in a phosphor screen for 24 h and developed on a phosphor imager (Storm 840, GE Healthcare, Piscataway, NJ).

Hybridization signal intensity was determined utilizing ImageQuant software (GE Healthcare). Briefly, each array was compared with negative control arrays utilizing the respective protocols without any starting RNA. Expression of TC amplified RNA bound to each target minus background was expressed as a ratio of the total hybridization signal intensity of the array (a global normalization approach). Global normalization effectively minimizes variation because of differences in the specific activity of the synthesized probe and the absolute quantity of the probe.²⁹

In situ hybridization

In situ hybridization was performed to validate our single cell expression findings. *In situ* hybridization was performed on 8 μ m paraffin-embedded nbM sections from Stage II and Stage IV CTE cases following the manufacturer's instructions. Customized probe of CLCN5 (Cat. No. 300031, Accession No. NM_001127899.3; Advanced Cell Diagnostics, Newark, CA) was used for *in situ* hybridization using the RNAscope 2.5HD Assay-Brown Kit (Cat No. 322300; Advanced Cell Diagnostics, Newark, CA). The probe region covers 1164 bp spanning nucleotides 771–1934. Expression of dihydrodipicolinate reductase mRNA from *Bacillus subtilis* was used as negative control. Images were obtained using a Nikon Eclipse microscope.

Statistical analysis

Demographic, clinical characteristics and cell counts were evaluated across pathological CTE Stages II, III, and IV using a one way analysis of variance (ANOVA) and the Kruskal–Wallis test followed by Holm–Sidak and Dunn's post-hoc test for multiple comparisons as appropriate with statistical significance set at 0.05 (two tailed).

Relative changes in total hybridization signal intensity and in individual mRNAs were analyzed by one way ANOVA with a Holm–Sidak post-hoc method for multiple comparisons. False discovery rates were also estimated as described previously.^{29,40} Expression levels were analyzed and clustered using bioinformatics and graphics software packages (GeneLinker Gold, Improved Outcomes Inc., Kingston, ON). Expression levels of select transcripts were correlated with clinical pathological variables using a Pearson test. The level of statistical significance was set at $p < 0.05$.

Results

Case demographics

The demographics of these 17 cases categorized as CTE Stage II, III, or IV^{1,3} have been previously published⁴ (see Table 1). Briefly, Stage II cases ($n=6$; mean age 44.2 ± 20.0) included three NFL

FIG. 1. Box plot showing the percentage of pS422-positive relative to total p75^{NTR}-positive nucleus basalis of Meynert (nbM) neurons in chronic traumatic encephalopathy (CTE) Stages II, III, and IV. Percentage of pS422-positive neurons was significantly increased in Stage IV compared with Stage II but not Stage III (Kruskal–Wallis follows Dunn's post-hoc test; $*p < 0.01$).

FIG. 2. Low power images showing pre (A, C, E) and post (B, D, F) laser capture microdissection (LCM) extraction of pS422-positive nucleus basalis of Meynert (nbM) neurons in a chronic traumatic encephalopathy (CTE) stage II, III, and IV subject. Black ovals outline the location of pS422-positive nbM after LCM retrieval (B, D, E). All sections were counterstained with Cresyl Violet. Scale bar = 50 μ m.

football players, one college football player, one semiprofessional football player, and one high school football player/wrestler/pole vaulter. Stage III cases ($n=5$; mean age 54.8 ± 9.3) were all NFL football players. Stage IV cases ($n=6$; mean age 75.0 ± 6.8) included five NFL football players, of which two were also military veterans, and a professional boxer. The groups differed by age ($p=0.008$), with Stage IV cases significantly older than Stage II cases, but no differences were found when subjects were compared by age to the time at which sport began, years of play, age at symptom onset, time interval between retirement and symptom onset, and interval between symptom onset and death (Table 1). As we reported previously,⁴ age when sport began was correlated with age at symptoms onset ($r=0.60$, $p<0.008$), greater interval between symptom onset and death ($r=0.48$, $p<0.04$), and age at death ($r=0.72$, $p<0.0003$). Age at symptom onset was related to increased interval between symptom onset and retirement from sport ($r=0.85$, $p<0.00001$), and with age at death ($r=0.71$, $p=0.0005$). Moreover, age at death was associated with longer intervals from symptom onset to either retirement from sport ($r=0.52$, $p=0.02$) or death ($r=0.64$, $p=0.003$).

Pathological evaluation

The present CTE subjects displayed other pathologies including: 13/18 (72%) with pTDP-43 inclusions, 6/18 (33%) with diffuse A β plaques, 3/18 (17%) with neuritic A β plaques, and 1/18 (6%) with Lewy body disease. Comorbid pathologies were more common in older subjects with a higher CTE stage.¹

Quantitation of the ratio of pS422 compared with p75^{NTR} nbM neurons

Quantitative analysis of pS422 and p75^{NTR} immunolabeled neurons within the nbM revealed that the percentage of pS422-positive neurons significantly increased in Stage IV compared with

Stage II, but not Stage III (Kruskal–Wallis with Dunn's post-hoc test, $p<0.001$) (Fig. 1).

Gene expression of pS422 immunopositive nbM neurons

Custom-designed microarrays were used to compare gene expression profiles in pS422 immunopositive nbM neurons among CTE cases displaying neuropathological stages II, III, and IV (Fig. 2). Quantitative analysis revealed that 10/576 total gene transcripts were significantly altered in between CTE stages (Table 2, Fig. 3). Gene ontology category classification included cholinergic and monoaminergic metabolism, endocytosis, intracellular signaling, and cytoskeleton/tau-related biology (Table 2). The nicotinic cholinergic receptor subunit β -2 (CHRN2) (Fig. 3A; Holm–Sidak post-hoc test, $p=0.03$), monoaminergic enzymes catechol-O-methyltransferase (COMT) (Holm–Sidak post-hoc, $p=0.038$) and dopa decarboxylase (DDC) (Holm–Sidak post-hoc test, $p=0.005$) transcripts were decreased in CTE Stage III compared with Stage II. Expression levels for calpain 2 (CAPN2), a cytoplasmic protease capable of cleaving tau, were downregulated in CTE Stage II compared with Stage III (Fig. 3B; one way ANOVA, Holm–Sidak post-hoc, $p<0.001$), and upregulated in CTE Stage IV compared with Stage III ($p=0.004$). Microtubule-associated protein 2 (MAP2) mRNA was upregulated in CTE Stage IV compared with Stage II (Fig. 3B; Holm–Sidak post-hoc, $p=0.019$). Cell homeostasis- and neurotransmission-related chloride channels CLCN4 and CLCN5 expression levels were downregulated in CTE Stage IV compared with Stage II (Fig. 3C; one way ANOVA, Holm–Sidak post-hoc, $p<0.05$), and only CLCN5 was significantly downregulated in CTE Stage IV compared with Stage III (Fig. 3C; Holm–Sidak post-hoc, $p<0.05$). The scaffolding protein caveolin 1 (CAV1) and cortical development/cytoskeletal element lissencephaly 1 (LIS1) transcripts were also decreased in CTE Stage IV compared with Stage III (Fig. 3C; Holm–Sidak post-hoc, $p<0.05$). The intracellular signaling cascade member adenylate cyclase 3 (ADCY3) was downregulated in CTE Stage

TABLE 2. SUMMARY OF GENES ALTERED IN nbM pS422 NEURONS ACROSS CTE STAGES

Gene abbreviation	Group	CTE Stage II	CTE Stage III	CTE Stage IV	p value	Pairwise comparison (p value)	Function
CLCN4	CH	4.11 ± 0.35^a	3.61 ± 0.43	3.54 ± 0.27	0.026	II > IV (0.037)	Cell homeostasis
CLCN5	CH	6.99 ± 0.87	6.96 ± 0.94	5.7 ± 0.47	0.025	II > IV (0.045) III > IV (0.044)	Cell homeostasis
CAV1	CYT	4.21 ± 0.33	4.67 ± 0.82	3.57 ± 0.46	0.018	III > IV (0.017)	Endocytosis
ADCY3	GP	1.70 ± 0.07	1.75 ± 0.10	1.54 ± 0.11	0.005	II > IV (0.016) III > IV (0.006)	Intracellular signaling
LIS1	DV	5.20 ± 0.26	5.62 ± 0.45	4.85 ± 0.38	0.012	III > IV (0.011)	Cortical development cytoskeleton
CHRN2	CHOL	3.67 ± 0.59	2.87 ± 0.41	3.18 ± 0.28	0.030	II > III (0.031)	Neurotransmission
COMT	MONO	3.01 ± 0.19	2.61 ± 0.24	2.92 ± 0.26	0.033	II > III (0.038)	Neurotransmission
DDC	MONO	4.66 ± 0.30	3.82 ± 0.37	4.30 ± 0.40	0.007	II > III (0.005)	Neurotransmission
MAP2	CYT	1.48 ± 0.11	1.66 ± 0.15	1.73 ± 0.15	0.018	II < IV (0.019)	Cytoskeleton tau
CAPN2	PROT	5.59 ± 0.38	4.50 ± 0.33	5.31 ± 0.35	<0.001	II > III (<0.001) IV > III (0.004)	Cell metabolism tau

^aMean $\pm$ SD (arbitrary units)

CH, monovalent and divalent channels; CHOL, cholinergic neurotransmission; CTE, chronic traumatic encephalopathy; CYT, cytoskeletal elements; DV, development-related elements; GP, G-protein subunits; MONO, monoamine neurotransmission, nbM, nucleus basalis of Meynert; PROT, protease-related markers.

FIG. 3. Linear representation showing the mean values of the gene expression level changes for neurotransmission (DDC, CHRN2, COMT) (A), cytoskeleton/cell metabolism (CAPN2, LIS1, MAP2) (B), and cell homeostasis/endocytosis and intracellular signaling (CLN5, CLN4, CAV1, ADCY3) (C) transcript across CTE stages. Note that MAP2 and CAPN2 mRNA levels were upregulated in CTE Stage IV compared with Stages II ($p=0.019$) and III ($p=0.004$), respectively, whereas the expression of the other genes was downregulated during the pathological progression of CTE (one way analysis of variance [ANOVA]). Holm–Sidak comparisons indicated by: *II vs. III; #II vs. IV, and \$III vs. IV. $p<0.05$.

IV compared with Stages II (Fig. 3C; Holm–Sidak post-hoc, $p<0.016$) and III (Fig. 3C; Holm–Sidak post-hoc, $p<0.006$). Table 3 shows examples of 10 randomly chosen genes (β actin [ACTB], glutamate receptor 1 [GRM1], amyloid precursor [A4] protein 1 [APLP1], heat shock protein [DNAJA1], synapsin 1 [SYN1], bcl2-related protein [BAK1], potassium channel [KCNA1], galanin peptide [GAL], NGFI-A binding protein 1 transcription factor [NAB1] and integrin α 1 [ITGA1]) displaying stable transcript levels in nbM pS422 positive neurons across CTE stages (one way ANOVA, $p>0.05$).

Gene expression in non-tangle bearing p75^{NTR} immunopositive neurons across CTE stages

Evaluation of the expression levels of those transcripts altered in pS422-containing neurons remained stable within non-tangle-bearing p75^{NTR} immunopositive nbM neurons across CTE stages (Fig. 4).

In situ hybridization

In situ hybridization of the CLCN5 gene was performed to validate our single-cell gene array findings. We found that CLCN5 expression was less abundant in CTE Stage IV compared with Stage II cases (Fig. 5) confirming our findings by custom-designed array analysis.

Expression level correlations

Transcript levels of CHRN2, COMT, and DDC in pS422-immunolabeled neurons showed a strong positive correlation with each other across CTE stages (Fig. 6A, B; Pearson correlation, coefficient [r] >0.7 , $p<0.001$). CAV1 correlated positively with ADCY3 (Fig. 6C; $r=0.5$, $p=0.03$) and LIS1 (Fig. 6D; $r=0.8$, $p<0.0001$) transcript levels across CTE stages. MAP2 transcript levels showed a weak negative correlation with CAPN2 ($r=-0.5$, $p<0.04$), whereas chloride channel CLCN4 and CLCN5 mRNA levels did not correlate with each other across CTE stages. Notably, only increases in MAP2 transcript levels were positively associated with age at which sport began (Fig. 6E; $r=0.5$, $p=0.04$), whereas CHRN2 transcripts levels were negatively associated with years played across the CTE stages (Fig. 6F; $r=-0.5$, $p=0.03$).

Discussion

The present study used single population gene expression profiling to examine the molecular alterations in tau-positive nbM neurons obtained from athletes and military persons who had antemortem brain trauma. Postmortem neuropathological analysis was performed on brain sections immunostained for tau and other pathological hallmarks of neurodegenerative disorders by the Brain Trauma Laboratory at VA Boston Healthcare System.⁴¹ Based on the distribution of tau profiles, the cases were categorized as: Stage II, Stage III, or Stage IV.^{3,41} Single population fingerprinting revealed significant dysregulation of select genes in several relevant gene ontology categories, including neurotransmitter, cell metabolism and homeostasis, and intracellular signaling and cytoskeletal elements within nbM neurons containing the tau pre-tangle marker pS422 between CTE stages.

Here we report a reduction in the cholinergic neurotransmitter-associated transcript CHRN2, which encodes the acetylcholine receptor subunit β 2, within nbM neurons in CTE Stage III compared to with Stage II. These findings are consistent with clinical pathological investigations demonstrating cholinergic-related deficits post-TBI in humans, including reduced ChAT activity and immunoreactivity within nbM neurons,¹² as well as downregulation of vAChT, and AChE.^{12,42} Although we did not find alterations in genes related to any of these cholinergic markers, perhaps it is possible that the reduced CHRN2 expression in CTE Stage III compared with Stage II indicates that the onset of defects in chemical transmission across synapses occurs later in the disease process. Although CHRN2 has not been linked to TBI previously, studies suggest that CHRN2 may be a candidate gene for neurodegenerative and neuropsychiatric disorders including schizophrenia⁴³ and AD.^{44,45} Interestingly, we reported an upregulation of the α 7 subunit of the

TABLE 3. EXAMPLE OF STABLE GENE TRANSCRIPTS IN nbM pS422 POSITIVE NEURONS ACROSS CTE STAGES

Gene abbreviation	Group	CTE Stage II	CTE Stage III	CTE Stage IV	p value	Pairwise comparison (p value)	Function
ACTB	CYT	2.29 ± 0.28 ^a	2.49 ± 0.22	2.51 ± 0.18	0.24	–	Cytoskeleton
GRM1	GLUR	2.23 ± 0.29	2.46 ± 0.21	2.43 ± 0.17	0.22	–	Neurotransmission
APLP1	AD	2.14 ± 0.34	2.20 ± 0.35	2.09 ± 0.34	0.86	–	Cell metabolism β-amyloid
DNAJA1	SR	2.50 ± 0.34	2.65 ± 0.29	2.55 ± 0.26	0.69	–	Cell metabolism
SYN1	SYN	6.54 ± 1.83	6.03 ± 1.65	6.16 ± 2.19	0.88	–	Synaptogenesis
BAK1	CD	2.63 ± 0.39	2.74 ± 0.27	2.64 ± 0.23	0.77	–	Cell metabolism
KCNA1	CH	2.54 ± 0.13	2.45 ± 0.20	2.44 ± 0.13	0.52	–	Cell homeostasis
GAL	PEP	1.86 ± 0.20	2.03 ± 0.32	1.99 ± 0.39	0.63	–	Neuromodulator
NAB1	TF	2.86 ± 0.45	3.14 ± 0.52	2.96 ± 0.40	0.56	–	Cell homeostasis
ITGA1	CYT	3.76 ± 0.29	3.66 ± 0.37	3.57 ± 0.33	0.64	–	Cell homeostasis

^aMean ± SD (arbitrary units).

AD, Alzheimer's disease related genes; CD, cell death-related genes; CH, monovalent and divalent channels; CTE, chronic traumatic encephalopathy; CYT, cytoskeletal elements; GLUR, glutamatergic neurotransmission; nbM, nucleus basalis of Meynert; PEP, peptides and peptide receptors; SR, stress response; SYN, synaptic-related markers; TF, transcription factor.

nicotinic receptor (CHRNA7) expression⁴⁰ and, recently, a down-regulation of the CHRNA2⁴⁶ in nbM neurons during the progression of AD. These data suggest that the cholinergic neurotransmission fingerprint of brain trauma resembles, in part, that seen in dementia.

The reduction in COMT and DDC expression within the nbM neurons in CTE Stage III compared with Stage II that was somewhat unexpected, as both genes are mainly associated with dopaminergic metabolism. On the other hand, dopamine plays a role in mood disorders, which are often reported in cases of CTE. Of interest are findings from immunohistochemical studies in primates, including humans, indicating that cells in the nbM may produce dopamine.^{47–50} Recently, we found a dysregulation of select dopaminergic receptor genes (DRD1 and DRD4) in tau-containing nbM neurons in AD (Tierman and coworkers, in preparation), which is further indication of changes in gene classes related to the dopaminergic metabolism within nbM neurons in CTE and AD.

Expression of CAPN2, a calcium-activated protease implicated in a wide variety of brain-related functions, including the regulation of cell death pathways and synaptic plasticity, was down-regulated in CTE Stage II compared with Stage III, and upregulated in Stage IV compared with Stage III.^{51–54} Numerous *in vivo* and *in vitro* investigations support the involvement of calpains in various trauma-induced disorders, including TBI.^{55–57} Rodent models of cortical brain trauma indicate that various calpain markers are elevated early and then decreased post-TBI. For example, in septohippocampal neuron cultures, mechanical injury induced a persistent elevation in calpain-mediated breakdown products as well as fragmented and beaded dendrites consistent with apoptotic cell death.⁵⁸ TBI also produces a loss of the cytoskeletal protein MAP2 marked by early changes in dendritic and cellular protein labeling following controlled cortical impact in rodent brain.^{59–61} We report upregulation of MAP2 expression in the late pathological stage of CTE, suggesting that an increase in MAP2 mRNA levels may be an attempt to compensate for calpain-induced neuronal toxicity.⁶² Because activated calpain cleaves MAP2, resulting in an alteration in neuronal architecture,⁵⁴ attenuating calpain abundance and/or activity may modify the molecular pathobiology leading to CTE. Consistent with this hypothesis, targeted gene inactivation of calpain-1 was shown to suppress

cortical degeneration in calpain-1 null mice post-TBI.⁵⁷ Moreover, human tau accumulation inhibits nicotinic acetylcholine receptor α4 by the activation of calpain-2, indicating that intracellular tau causes cholinergic impairments in neuronal cells.⁶³ Perhaps targeted inhibition of calpains may offer a novel therapeutic target not only for tau dysregulation, but also for cholinergic deficits in TBI/CTE.

CAV1 encodes the scaffolding caveolin1 (cav-1) protein found in the caveolae plasma membrane in endocytic processes was downregulated in nbM neurons during CTE progression. Similarly, we reported a downregulation of the endocytic-related CAV2 transcript in nbM neurons in AD.⁶⁴ These findings suggest that caveolin and endocytic processes in nbM neurons are pathologically compromised post-brain trauma as well as in AD.⁶⁴ Other studies indicate that cav-1 protein levels in the adult cortex are increased in the endothelium for several days after cold cortical injury,^{65,66} brain ischemia, and spinal cord injury.⁶⁷ A similar response was reported following controlled cortical impact in juvenile rats.^{68,69} Interestingly, astrogliosis and microgliosis are increased in the brains of cav-1 knockout mice post brain insult, suggesting that cav-1 may play a role in the activation of a neuroinflammatory response following brain injury.^{70,71} Cav-1 knockout mice exhibit enhanced anxiety, impaired spatial memory, and altered cholinergic function similar to findings reported in patients with CTE.⁷² In addition, a beneficial role for cav-1 delivery has been demonstrated in experiments using cav-1 knockout mice.⁶⁸ Whether a beneficial affect would be true in humans remains to be determined.

Our findings of a downregulation of ADCY3 expression suggest a defect in the ability of nbM neurons to catalyze the formation of the secondary messenger cyclic adenosine monophosphate (cAMP), which is associated with neuronal survival, and is reduced for many weeks following TBI.⁷³ Restoration of the cAMP pathway improves recovery after brain injury, possibly by stimulating transcription of cell survival genes,^{74–77} which are reduced in the hippocampus and parietal cortices following fluid-percussion induced brain injury.⁷³ Rolipram, a selective inhibitor of the cAMP-degrading drug phosphodiesterase IV, restored cAMP in the hippocampus, improved neuronal survival compared with vehicle controls, reduced total tau, and improved cognitive performance in a tau

FIG. 4. Histogram illustrating gene expression for neurotransmission (DDC, CHRN2, COMT) (A), cytoskeleton/cell metabolism (CAPN2, LIS1, MAP2) (B), and cell homeostasis/endocytosis and intracellular signaling (CLN5, CLN4, CAV1, ADCY3) (C) transcripts in non-tangle-bearing nucleus basalis of Meynert (nbM) p75^{NTR}-positive neurons across chronic traumatic encephalopathy (CTE) stages. Note that mRNA levels were unchanged in non-tangle-bearing neurons across the CTE stages (one way analysis of variance [ANOVA]). $p < 0.05$.

mutant mouse.^{78,79} Perhaps this drug may be useful for the clearance of putative toxic tau isoforms, and should be considered as a treatment approach for human CTE.

The decrease in expression of LIS1 in CTE Stage IV compared with Stage III has not been reported in the pre-clinical or clinical literature. LIS1 is a member of the WD40-repeat family of proteins, and was the first gene identified with neuronal migration defects in

FIG. 5. CLCN5 *in-situ* hybridization images showing differential labeling of nucleus basalis of Meynert (nbM) neurons from chronic traumatic encephalopathy (CTE) Stage II (A) and Stage IV (B) subjects. Note that there are many more CLCN5 mRNA copies (brown dots; arrows) in CTE Stage II than in CTE Stage IV. Sections were counterstained with hematoxylin. Scale bar = 10 μ m.

the developing and adult brain.^{80,81} The functional significance of a reduction in LIS1 expression in CTE tau-positive nbM neurons remains to be determined. However, studies indicate a strong relationship between LIS1 and microtubule regulation, microtubule-based motor proteins,⁸² preservation of normal microtubule network organization,⁸³ neuritic structure, axonal transport, and synaptic function.^{81,84} Taken together, these findings suggest that the down-regulation of the LIS1 gene may be associated with neuronal architecture alterations in advanced CTE.

CLCN4 and CLCN5 are members of the CLCN family of voltage-dependent chloride channel genes.⁸⁵ Encoded CLCN proteins are mainly localized to the endosomal membrane, and subserve a wide range of cellular functions, including the regulation of electrical excitability of neurons.⁸⁶ A search of the literature failed to reveal studies showing an interaction between the CLCN4 and CLCN5 genes following brain trauma. Whether the reduction in CLCN5 expression found in the present study is related to mechanisms of cellular apoptosis in nbM neurons in CTE remains to be investigated.

Notable caveats related to the current study are acknowledged. There are currently a limited number of neuropathologically verified CTE cases, effectively limiting the number of basal forebrain samples that we could analyze in this study. The pathological diagnosis of CTE and subsequent staging is performed on paraffin-embedded tissue blocks accrued from donated brains,³ and frozen brain tissue unfortunately is rarely available. A major caveat related to the lack of frozen basal forebrain tissue is the inability to perform quantitative polymerase chain reaction (qPCR) for validation of the current single cell expression data. To address this obstacle, we collaborated with Advanced Cell Diagnostics to generate a customized *in situ* hybridization probe to independently assess CLCN5 expression. Using this probe, we demonstrated that CLCN5 was less abundant in nbM neurons in CTE Stage IV than in Stage II, validating our custom-designed microarray findings. Another problem is that non-CTE control cases were not available for comparison, as cohorts of age-matched military personnel and/or athletes without TBI/CTE have not been generated to date. Because non-CTE control subjects are not available, we analyzed our data by neuronal chemical phenotype across CTE stages. This scheme allowed only for an analysis of expression profiling in ps422-immunoreactive tau nbM neurons compared and contrasted across CTE stages. Future studies will attempt comparisons of dual labeled neurons when larger cohorts of CTE and control basal forebrain samples are available.

FIG. 6. DDC transcript levels correlated positively with COMT (A) and CHRN2 (B) expression in within the pS422-positive nucleus basalis of Meynert (nbM) across chronic traumatic encephalopathy (CTE) stages. Likewise, CAV1 mRNA levels correlated positively with ADCY3 (C) and LIS1 (D) levels in CTE. MAP2 (E) transcript levels correlated positively with the age at which sport began, whereas CHRN2 (F) correlated negatively with years of nplay in CTE. Stage II, red circles; Stage III, blue circles; Stage IV, green circles.

Because this is the first single population expression study of human tau-positive nbM neurons obtained from post-TBI subjects, it is difficult to compare and contrast the current findings with other published reports. For example, a genomic investigation of biopsied frontal and temporal cortical tissue obtained during surgery from TBI patients and homogenized prior to microarray analysis detected 1200 genes, with ~8% of them showing differential

regulation.²³ Although there was no overlap between the specific transcripts found in the present study, the broader gene ontology categories showing mRNA alterations displayed similarities, including transcriptional regulation, energy metabolism, and signal transduction. A key feature of the present single population microarray analysis study using specific phospho-tau labeled vulnerable nbM neurons is that it allows the parsing of gene expression

signatures from adjacent neuronal and non-neuronal cell types. Further, these findings provide a glimpse into critical mechanistic information at the cellular molecular level related to progressive CTE, which may be used as the infrastructure for finding novel diagnostic and treatment strategies.

Conclusion

In summary, we found that select genes related to cholinergic/monoaminergic neurotransmission, cell survival/death, and signal transduction, among other relevant gene ontology categories, are differentially regulated within nbM neurons during distinct pathological CTE stages. Interestingly, few of the specific transcripts dysregulated in CTE nbM neurons have been reported in single cell array studies of this cell population during the progression of AD.^{25,87,88} In AD, we found significant changes in a myriad of genes including synaptic, endosomal-lysosomal autophagic, and neurotrophin receptor categories, which is notable, because both AD and CTE contain NFT pathology within the nbM.^{4,24–28,88} The differences in altered expression profiles may be related to different mechanisms underlying nbM degeneration in each of these neurological disorders as well as the greater access to AD and age-matched control cases. Age may be a factor as CTE subjects are younger than many AD cases. Neuronal degeneration in CTE results from excessive concussive impacts over time, where AD onset is most likely driven by a confluence of intracellular events and possibly the aging process itself. Trauma also cannot be excluded from AD pathogenesis, although most sporadic AD patients are not professional athletes or military personnel with brain injuries. In conclusion, we provide an initial assessment of tau-containing nbM neurons across CTE stages, and demonstrate select alterations in several genes that may be useful for transcriptional-based drug discovery purposes.

Acknowledgments

This material is based on work supported by the United States Army Medical Research and Materiel Command and from the United States Department of Veterans Affairs (Chronic Effects of Neurotrauma Consortium) under Award No. W81XWH-13-2-0095. The United States Army Medical Research Acquisition Activity, 820 Chandler Street, Fort Detrick MD 21702-5014 is the awarding and administering acquisition office. Any opinions, findings, conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the United States government, or the United States Department of Veterans Affairs, and no official endorsement should be inferred. An award from the Department of Veterans Affairs (No. I01 RX001774), National Institute of Neurological Disorders and Stroke, National Institute of Biomedical Imaging and Bioengineering (U01NS086659-01), National Institute of Aging Boston University AD Center (P30AG13846, supplement 0572063345-5), the Veterans Affairs Biorepository (CSP 501), the National Operating Committee on Standards for Athletic Equipment, the Concussion Legacy Foundation, the Andlinger Foundation and the World Wrestling Entertainment and the National Football League supported this work.. The authors gratefully acknowledge the use of the resources and facilities at the Edith Nourse Rogers Memorial Veterans Hospital (Bedford, MA) and Barrow Neurological Institute Department of Neuropathology (Phoenix, AZ).

Author Disclosure Statement

No competing financial interests exist.

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