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Expression of HIV envelope protein in the human central nervous system

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Objective/design: The HIV envelope glycoprotein gp120 contributes to neuronal damage that could lead to HIV-mediated neurocognitive disorder. However, it is debated whether gp120 could promote direct neurotoxicity to synapses as it may not be present in the central nervous system of people with HIV (PWH) on antiretroviral therapy (ART). This study sought to establish whether gp120 is expressed in the human central nervous system in the pre-ART and post-ART era.

Methods: We utilized real-time PCR to detect the envelope mRNA in postmortem caudate nucleus from pre-ART era. HIV-negative samples were used as controls. We then used RNAscope fluorescence multiplex to identify which cells express the envelope mRNA. To determine whether gp120 is present despite ART, we analyzed cerebrospinal fluid (CSF) samples from PWH on ART using mass spectrometry.

Results: Using PCR, we detected the envelope mRNA in the caudate nucleus from PWH with neurocognitive impairment but not in control samples. With RNAscope, we detected the envelope mRNA in microglia and astrocytes in the frontal cortex and caudate nucleus from HIV-positive tissue. With mass spectrometry, we identified a gp120 peptide in the CSF of PWH on antiretroviral therapy ($n = 20$) but not in control participants ($n = 7$).

Conclusion: Our data suggest that despite antiretroviral therapy, gp120 is expressed and likely released by infected cells, suggesting that gp120 could be one of the key factors in the neuropathology observed in PWH.

Graphical abstract: http://links.lww.com/QAD/D470

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Introduction

Human immunodeficiency virus type 1 (HIV) invades the central nervous system (CNS) soon after the initial infection of the immune system. HIV infection of the CNS, and in particular the brain, can lead to HIV-associated neurocognitive disorders (HAND). HAND refers to a spectrum of neurocognitive impairments that include asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorders (MND), and HIV-

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associated dementia (HAD). The use of antiretroviral therapy (ART) has shown positive outcomes in reversing and preventing HAD, but a substantial proportion of people with HIV (PWH) still exhibit mild neurocognitive impairments [1]. ART does not eradicate HIV from the CNS. Currently, there are no therapeutics available to block the production of HIV transcripts from the integrated provirus. Thus, some PWH on ART have detectable levels of HIV RNA/DNA in their white matter across various brain areas as well as in the cerebrospinal fluid (CSF), even when it is undetectable in blood [2–4]. Moreover, the CNS can serve as a reservoir for ongoing HIV replication [5,6] with certain CNS cells harboring persistent infection.

Neurological alterations seen in HAND include loss of synapses [7–9], and a reduction of gray and white matter volume [10,11]. However, little is known about the mechanisms leading to the selective vulnerability of synapses in HAND considering that neurons are not infected. HAND is characterized by HIV encephalitis (HIVE) and HIV leukoencephalopathy. HIVE is an inflammatory response with disseminated infiltrates of lymphocytes, macrophages, and multinucleated giant cells, while leukoencephalopathy implies bilateral diffuse loss of myelin in the hemispheric white matter, along with astrocytosis and microglial infiltration. However, HIVE has become less common in HAND after the introduction of ART and is not a necessary feature of all HAND pathogenesis. In fact, HIVE is rarely seen in ANI and/or MND [12]. Thus, HAND cannot be a consequence of HIVE only. Aging [13], ART [14], and various comorbidities [15] appear to exacerbate the frequency of neurocognitive deficits. Moreover, HIV-infected cells in the CNS, which include resident (microglia and astrocytes), and nonresident macrophages, produce HIV virions, as well as the viral proteins Tat, gp120, and Nef [16–19]. PWH with cognitive impairment have detectable levels of these proteins in their brains or CSF despite ART [20,21]. Tat and gp120 exhibit a unique mechanism of neurotoxicity because in addition to promoting the release of pro-inflammatory cytokines from microglia and astrocytes [22,23], they appear to be neurotoxic via their interactions with neuronal receptors or their endocytosis into neurons [24,25]. Thus, the combination of neuroinflammation and exposure to viral proteins may cause pruning of synapses.

The exact mechanisms of such neurological abnormalities are not entirely known. Some individuals do not develop HAND, suggesting that not all PWH develop neuroinflammation. Yet, evidence has shown that infected cells can release gp120 and other viral proteins to cause neuronal degeneration. Intriguingly, experimental data indicate that gp120 can promote dendritic pruning even in the absence of microglia or neuroinflammation [26,27]. Thus, the host response to these viral proteins might be a key factor explaining the neuropathological scenario seen in HAND. However, an important question raised in the field is whether gp120, the product of the envelope protein (env), is present in the CNS in the ART era. To establish the presence of gp120 in the brain, we examined brain sections from HAND individuals without ART for the env messenger RNA (mRNA) using a sensitive RNAscope. Moreover, we used mass spectrometry (MS) to detect peptides of gp120 in the CSF of PWH under ART. Here, we present evidence that gp120 mRNA and protein are expressed in brain and CSF, respectively, of PWH.

Methods

Human samples

Postmortem tissue of PWH not treated with ART were obtained from the National NeuroAIDS Tissue Consortium. A total of six samples from PWH with or without neurocognitive impairment were used. Control samples (HIV-negative) with cognitive impairment were obtained from the Alzheimer's disease brain bank at Georgetown University Medical Center. All samples were coded to protect identity.

Twenty-seven CSF samples from seven study persons without HIV, 10 PWH without cognitive impairment, and 10 PWH with cognitive impairment were obtained from the NIH-Neuro-HIV Research Consortium (NIH/NINDS, Bethesda, Maryland, USA). All HIV-positive CSF were from virally controlled ART-treated study persons. Their diagnosis/characteristics are described in Table 1. All participants consented to the study. Samples were coded. The HIV long LTR copies/ml in each sample is included to show whether gp120 can be detected even without many copies of HIV. Twenty microliters of 10% SDS was added to 200 μl of each sample (final: 1% SDS) and incubated for 30 min at room temperature (RT) to inactivate HIV.

Animals

Brain tissue from gp120 transgenic (tg) mice was used as a positive control for RNAscope. Mice were maintained in our facility housed under standard conditions with food and water *ad libitum* on a 12 h light/dark cycle. Mice were euthanized by intracardial exsanguination. Genotype was confirmed through an outsourced genotyping service (Transnetyx, Inc., Cordova, Tennessee, USA) from tail snips taken at time of weaning and at euthanasia. All studies were carried out following the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health and approved by the Georgetown University Animal Care and Use Committee.

Reverse transcriptase-PCR

mRNA was extracted using the NucleoSpin kit (cat No. 740955.50, Machery-Nagel, Alletown, Pennsylvania,

Table 1. Characteristics of cerebrospinal fluid study samples from the NIH-Neuro-HIV Research Consortium.

Sample ID	HIV status	Cognitive impairment	HAND diagnosis	Race	$CD4^+$	Viral load	HIV long LTR (copies/ml)	ART
1	HIV-	Not impaired	No HAND	W				
2	HIV-	Not impaired	No HAND	W				
3	HIV-	Not impaired	No HAND	W				
4	HIV-	Not impaired	No HAND	W				
5	HIV-	Impaired	ANI	AA				
6	HIV-	Impaired	ANI	AA				
7	HIV-	Impaired	ANI	AA				
8	HIV+	Not Impaired	No HAND	AA	740	<40	729.63	Bictegravir/tenofovir AF/emtricitabine
9	HIV+	Not impaired	No HAND	W	124	ND	–	Abacavir + lamivudine + etravirine
10	HIV+	Not impaired	No HAND	AA	348	<40	53.68	Abacavir + lamivudine + efavirenz
11	HIV+	Not impaired	No HAND	AA	493	ND	–	Efavirenz + emtricitabine + tenofovir
12	HIV+	Not impaired	No HAND	W	687	ND	0	Elvitegravir/cobicistat/tenofovir DF/emtricitabine
13	HIV+	Not impaired	No HAND	AA	994	<40	147.03	Abacavir + lamivudine + ritonavir + darunavir
14	HIV+	Not Impaired	No HAND	AA	450	<40	120.21	Bictegravir/tenofovir AF/emtricitabine
15	HIV+	Not impaired	No HAND	W	581	<40	–	Elvitegravir/cobicistat/TAF/emtricitabine
16	HIV+	Not impaired	No HAND	W	417	ND	218.79	Emtricitabine/tenofovir alafenamide + dolutegravir + darunavir/cobicistat
17	HIV+	Not impaired	No HAND	W	400	<40	134.83	Abacavir/dolutegravir/lamivudine
18	HIV+	Impaired	ANI	AA	1264	<40	1250.22	Efavirenz + emtricitabine + tenofovir DF
19	HIV+	Impaired	ANI	AA	1493	<40	0	Abacavir/dolutegravir/lamivudine
20	HIV+	Impaired	ANI	W	1030	<40	62.01	Abacavir/dolutegravir/lamivudine
21	HIV+	Impaired	ANI	W	314	<40	–	Emtricitabine/tenofovir alafenamide + dolutegravir
22	HIV+	Impaired	ANI	W	521	<40	100.39	Abacavir + efavirenz + ritonavir + fosamprenavir
23	HIV+	Impaired	ANI	AA	694	<40	40.96	Elvitegravir/cobicistat/tenofovir DF/emtricitabine + darunavir
24	HIV+	Impaired	HAD	W	292	<40	0	Abacavir/dolutegravir/lamivudine
25	HIV+	Impaired	HAD	AA	595	<40	0	Emtricitabine/tenofovir alafenamide + raltegravir
26	HIV+	Impaired	ANI	AA	103	500	118.56	Emtricitabine/tenofovir DF + darunavir/cobicistat
27	HIV+	Impaired	MND	AA	–	–	133.68	Abacavir + lamivudine + ritonavir + darunavir

Samples were coded to protect study person identity. Seven CSF samples were from study persons without HIV, four of which were not cognitively impaired, and three of which exhibit cognitive impairment. Ten CSF samples were from PWH without cognitive impairment, and 10 were from PWH with cognitive impairment. All HIV-positive CSF samples are from PWH treated with ART. The mean age in years was 55.8 (4.26) for the HIV-negative, not impaired group; 50.7 (4.49) for the HIV-negative, cognitively impaired group; 60 (7.75) for the HIV-positive, not impaired group; and 57.6 (3.3) for the HIV-positive, cognitively impaired group. The sex, expressed as the number of male individuals and percentage of total study persons, is $n = 3$ (75%) for the HIV-negative, not impaired group; $n = 2$ (67%) for the HIV-negative, cognitively impaired group; $n = 7$ (70%) for the HIV-positive, not impaired group; and $n = 5$ (50%) for the HIV-positive, cognitively impaired group. The HIV long LTR copies/ml show that gp120 can be detected even without many copies of HIV. –, data not available; AA, African American; ANI, asymptomatic neurocognitive impairment; HAD, HIV-associated dementia; HAND, HIV-associated neurocognitive disorder; MND, mild neurocognitive disorder; ND, none detected; W, white/Caucasian.

USA). Samples were quantified on a Nanodrop and normalized to 250 ng/μl. RT-PCR was performed using the SuperScript IV VILO Master Mix (cat No. 11766050 ThermoFisher Scientific, Waltham, Massachusetts, USA) and prepared according to the manufacturer's protocol. The resulting cDNA was quantified on a Nanodrop. Primers used to amplify a small conserved sequence of the env mRNA are described in Fig. 1. Temperatures for the PCR reaction were: 1 cycle at 96 °C for 2 min then a following 36 cycles at 94 °C 1 min, 59 °C 1 min 30 s, and 72 °C for 1 min 30 s. These cycles were then followed by one cycle of 72 °C for 7 min. The PCR products were run on a 1% Ultrapure agarose gel (cat no.16500-500, Invitrogen, USA) in 1× Tris-acetate-ethylenediaminetetraacetic acid buffer 12.5 μl of DNA gel stain (cat no. S33102, Invitrogen).

Fluorescent multiplex detection with RNAscope

RNAscope was performed with Fluorescent Detection Kit v2. (cat no. 323110, Advanced Cell Diagnostics Inc., Newark, California, USA) according to manufacturer's protocol for formalin-fixed paraffin-embedded tissue. Mouse brains were fixed in 10% neutral-buffered formalin, embedded in paraffin, dehydrated, sectioned at 5 μm. RNAscope mouse probe for env (cat no. 318281) was purchased from Advanced Cell Diagnostics Inc. and validated with positive and negative control probes for 2 h at 40 °C. The amplification steps were performed according to manufacturer's directions. Detection was performed by Opal Dye 570 (cat no. FP1488001KT, Akoya Biosciences, Marlborough, Massachusetts, USA) for gp120 and Opal Dye 620 (cat no. FP1495001KT, Akoya Biosciences,) for RbFox3. Brain sections were rinsed with PBS 3× and incubated for 5 min in PBS with 4′,6-diamidino-2-phenylindole (1 : 50 000, Sigma-Aldrich, St. Louis, Missouri, USA) for counterstained nuclei. Images were acquired on a Leica SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany) in z-stacks (40×/1.4 magnification, 1024 × 1024, 200 ms scanning speed, 0.3 μm/section, 1.2 μm stack total).

Following in-situ hybridization, sections were processed for immunohistochemistry. Briefly, following the blocking step with 5% bovine serum albumin (BSA) in PBS for

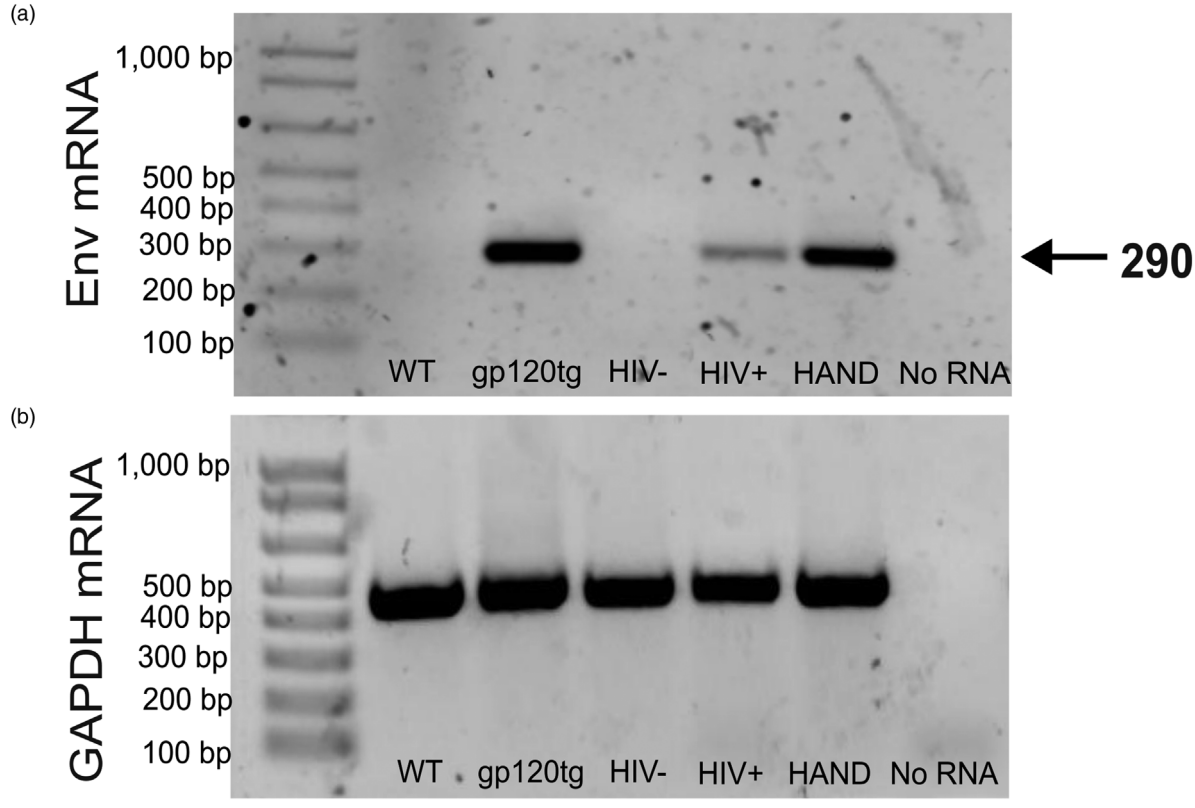

Fig. 1. The env messenger RNA is expressed in the human brain. (a) Primers were designed to amplify the messenger RNA (mRNA) encoding for the C5 domain of the env protein of HIV-LAV1 by RT-PCR. Primers were: forward (5′-ATCGTGTTGT-CAGTCCTGGTG-3′) and reverse (5′-CTGTTTGCAGCAGAGAAAGCC-3′). Total mRNA was extracted from postmortem caudate nucleus of study persons that were HIV negative (−), HIV+ with no cognitive impairment [viral load in the cerebrospinal fluid (CSF) and plasma: 5019 and 702 257 copies/ml, respectively], or HAD (viral load in the CSF and plasma: 2747 and 1249 copies/ml, respectively). mRNAs from wild type and gp120tg mice were used as negative and positive controls, respectively, to confirm that primers amplified the expected mRNA. (b) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as loading control. Left lane, molecular weight markers.

1 h at RT, posthybridized slides were incubated with an antibody against glial fibrillary acidic protein (GFAP) (1 : 500, EMD Millipore, Temecula, California, USA), ionized calcium binding adaptor molecule-1 (Iba1) (1 : 500, Wako Chemical USA, Richmond, Virginia, USA, or neuronal nuclear antigen (NeuN) (1 : 100, EMD Millipore) in 5% BSA in PBS overnight at 4 °C. Following three PBS washes, the slides were incubated with corresponding Alexa Fluor 488 secondary antibodies (1 : 2000; Molecular probes, Grand Island, New York, USA) for 2 h at RT. Images were acquired on a Leica SP8 confocal microscope (Leica microsystem).

Cells were imaged using a confocal microscope. Cell counts were performed using ImageJ (NIH, Bethesda, Maryland, USA). The number of GFAP-positive or Iba1-positive cells, and the number of GFAP/Env or Iba1/Env double-positive cells were manually counted across two or three sections per brain region. The number of double-positive cells was expressed as a percentage of the total GFAP-positive or Iba1-positive cell count.

Nanoscale liquid chromatography coupled to tandem mass spectrometry

Proteins (equal amount per CSF sample) were treated with dithiothreitol and iodoacetamide and loaded onto a S-Trap column (ProtiFi, LLC) following a published procedure [28]. Proteins were digested with sequencing-grade Lys-C/trypsin (Promega corporation, Madison, Wisconsin, USA) by incubation at 37 °C overnight. The resulting peptides were eluted and dried down with a SpeedVac (ThermoFisher Scientific).

Peptides were analyzed with a nanoAcquity UPLC system (Waters) coupled with Orbitrap Fusion Lumos mass spectrometer (ThermoFisher Scientific), as described previously [28]. Samples in 0.1% formic acid (FA) solution were loaded onto a C18 Trap column (Waters Acquity UPLC M-Class Trap, Symmetry C18, 100 A, 5 μm, 180 μm × 20 mm) and separated with an analytical column (Waters Acquity UPLC M-Class, peptide BEH C18 column, 300 A, 1.7 μm, 75 μm × 150 mm). A 150 min gradient was used for separation: 1% buffer B at 0 min, 5%

buffer B at 1 min, 22% buffer B at 90 min, 50% buffer B at 100 min, 98% buffer B at 120 min, 98% buffer B at 130 min, 1% buffer B at 130.1 min, and 1% buffer B at 150 min. MS data were acquired in data-dependent acquisition mode on the Orbitrap Fusion Lumos mass spectrometer using an ion spray voltage of 2.4 kV and ion transfer temperature of 275 °C. Mass spectra was recorded with Xcalibur 4.0. Advanced peak determination was on for MS analyses. The MS parameters were set as: detector type: Orbitrap; mass range: 375–1500 m/z; Orbitrap resolution: 120 000; scan range: 375–1500 m/z; RF lens: 30%; AGC target: standard; maximum injection time mode: auto; microscans: 1; charge state: 2–7; exclusion duration: 40 s; and cycle time: 3 s. HCD was used for MS/MS acquisition. MS/MS parameters were set as: isolation mode: Quadrupole; isolation window: 1.6 m/z; stepped collision energy of 22.5, 30, 37.5% was used. Detector type: Orbitrap; resolution: 30 000; normalized AGC target: 200%.

Data analysis

The MS raw data were processed using Proteome Discoverer (v2.4, ThermoFisher Scientific) and Sequest HT algorithm. Database search was done using a custom database containing protein sequence of HIV group M subtype B (isolate BH10, PO3375) and HIV human group M subtype B (isolate BRU/LAI, PO3377) based on the Universal Protein Resource (UniProt) [29]. Trypsin was the enzyme for cleavage. Two missed cleavages were allowed, and the minimum peptide length was set to seven amino acids. Variable modifications included oxidation (M) and protein N-terminal acetylation. Fixed modification included carbamidomethylation (C). MS and MS/MS ion tolerances were set at 10 ppm and 0.6 Da, respectively. The false-discovery rate (FDR) was estimated using the fixed value PSM validation. On the peptide level, the corresponding FDR was less than 1%. Mass spectra of peptides from gp160 were manually checked and annotated. Relative quantification of proteins in samples was performed based on intensity using unique and razor peptides. The normalization and scaling modes were set as total peptide amount and, on all average, respectively. Summed abundances of peptides were used to calculate protein abundances, by using pairwise *t*-test.

Graphical abstract and figures

The graphical abstract was created with Biorender (http://www.biorender.com). Inkscape was used to make figures.

Results

The env messenger RNA is detectable in postmortem tissue

To amplify the HIV env mRNA by PCR, we designed two primers, which amplify 290 base pairs of the C5 env domain [30]. To confirm the reliability and specificity of our detection method, we tested these primers on brain tissue from wildtype and gp120 transgenic (tg) mice [31]. These mice, which express the human gp120 under the GFAP promoter, have been characterized in terms of behavior, neuropathology [31], and age-related synaptic loss [32]. Both primers amplified the expected mRNA of about 290 bases in gp120tg mice only (Fig. 1a). We, therefore, used PCR and the C5 primer to amplify the env mRNA from postmortem human caudate nucleus of HAD. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as loading control (Fig. 1b). We detected the expected 290 bases band in HIV-positive tissue but not in HIV-negative tissue (Fig. 1a). There was more env mRNA in HAND caudate than in that of PWH with no cognitive impairment. However, the number of samples ($n = 2$, each) is too small for statistical analysis.

Histological analyses of gp120 mRNA

Commercially available antibodies against gp120 may exhibit nonspecific binding or the inability to detect low protein levels. Thus, we used in-situ hybridization to map the expression of the env mRNA in postmortem human brains, combined with immunohistochemistry (IHC) for specific cell markers. These include GFAP, an astrocytic marker, Iba-1, a microglia/macrophage-specific marker, and NeuN, a neuronal marker. First, we validated the specificity of the gp120 probe for RNAscope by comparing gp120 mRNA signal in brain sections from wildtype and gp120tg mice. We observed that, in the hippocampus of gp120tg mice, the gp120 probe produced a strong fluorescent signal, characterized by red dots (Supplementary Fig. 1a, http://links.lww.com/QAD/D471). The gp120 dots were localized in GFAP-positive cells (Supplementary Fig. 1b, http://links.lww.com/QAD/D471), indicating astrocytic expression and confirming that gp120 mRNA is driven by a GFAP promoter [31]. No hybridization for gp120 mRNA was observed in wildtype mice (Supplementary Fig. 1b, http://links.lww.com/QAD/D471), supporting the specificity of the gp120 mRNA probe used for RNAscope.

We then analyzed human post mortem caudate nucleus and frontal cortex for the env mRNA. These samples were from PWH without ART. We detected the env mRNA in the caudate nucleus and frontal cortex in both microglia (Fig. 2) and astrocytes (Fig. 3). No signal was detected in neurons (data not shown) or in HIV-negative samples (Fig. 3a). Most of the env mRNA was localized in Iba1-positive cells, indicating microglia cells (Fig. 2). However, the prevalence of env mRNA in microglia varies. In one sample, 71% of Iba1-positive cells in a given field were positive for env mRNA (Fig. 2), whereas in other samples, ~30% of Iba1-positive cells were positive for env mRNA. This was not unexpected, because in the brain, HIV preferentially infects microglia. Similarly, the percentage of astrocytes with env mRNA puncta varied

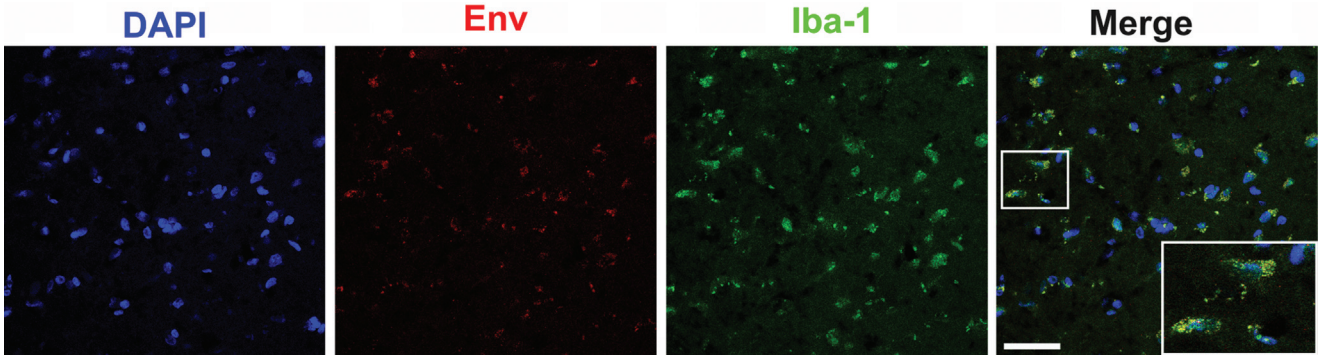

Fig. 2. Immunohistochemical localization of env messenger RNA in the human caudate. RNAscope was used to detect the env messenger RNA (mRNA) expression in HIV+ postmortem caudate nucleus [viral load in the cerebrospinal fluid (CSF) and plasma: 5019 and 702 257 copies/ml, respectively]. Shown are representative images for the env mRNA (red), Iba1 (green), and DAPI (blue). Merge shows the env mRNA within Iba1 positive cells (yellow). Merge includes a high-powered magnification field of view. Seventy-one percent of Iba1-positive cells in this field of view were positive for the env mRNA. However, the prevalence of env mRNA in microglia varies from 30 to 70%, depending on field of view and sample. Scale bar = 50 μm. DAPI, 4′,6-diamidino-2-phenylindole.

from 12 to 50% per field of view (Fig. 3e). The frontal cortex contained less env mRNA puncta when compared with the caudate nucleus (Fig. 3b and d); however, most of the cortical astrocytes positive for the env mRNA were clustered (Fig. 3c), suggesting activated astrocytes typically associated with HIVE. These results validate the notion that astrocytes can be a reservoir for HIV [5,33,34].

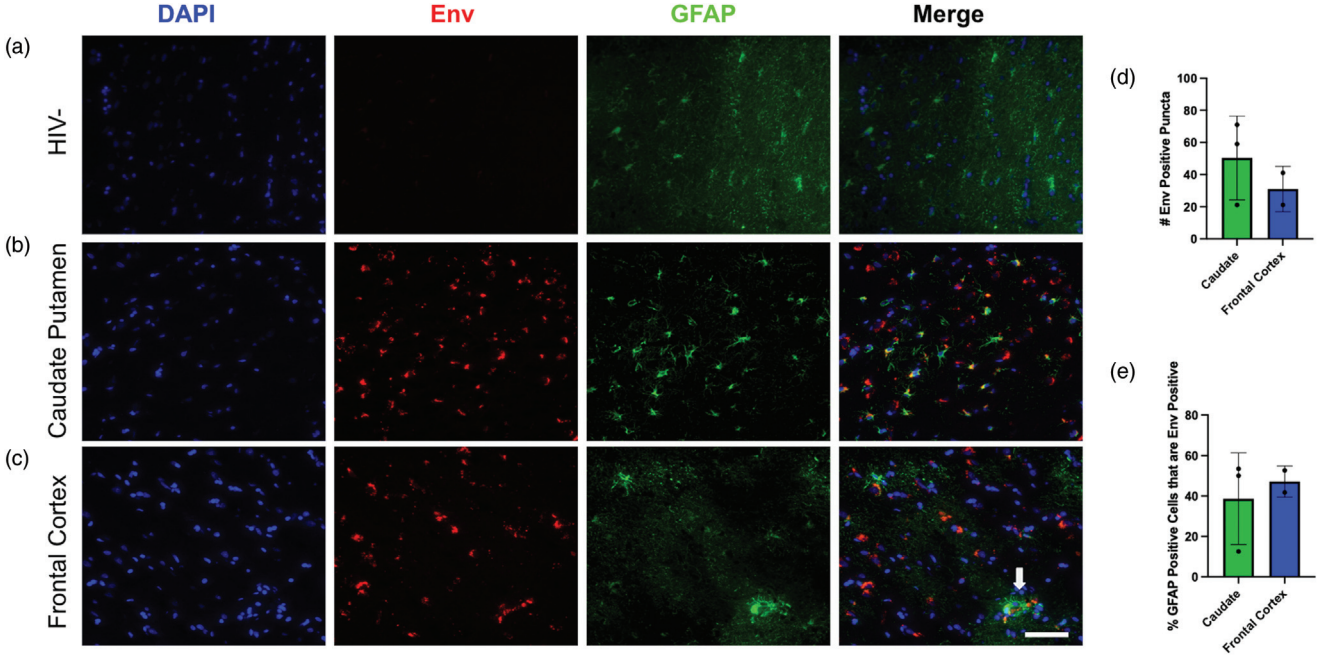

Fig. 3. The env messenger RNA is expressed in human astrocytes. RNAscope and immunohistochemistry were used to examine whether astrocytes of HIV-negative (a) and HIV-positive tissue (b and c) express the env messenger RNA (mRNA). (b and c) Representative images of sections analyzed for the env mRNA (red) and glial fibrillary acidic protein (GFAP) (green) immunohistochemistry in HIV+ postmortem caudate nucleus [viral load in the cerebrospinal fluid (CSF) and plasma: 5019 and 702 257 copies/ml, respectively] and the frontal cortex of a subject with HAD (viral load in the CSF and plasma: n/a and 505 903 copies/ml, respectively). Merge shows a number of yellow (red + green) puncta denoting colocalization of the env mRNA within astrocytes. White arrow denotes clustered astrocytes. Please note that in HIV-negative samples (a) no env mRNA was detected. (d) The mean number of env-positive puncta across three randomly chosen fields of view for the caudate putamen was 50.33, while the mean env-positive puncta for the frontal cortex was 31. (e) 38.5% of GFAP-positive cells, averaged across three randomly chosen fields of view, in the caudate are positive for env mRNA, and 47.15% of GFAP-positive cells in the frontal cortex are positive for env mRNA. Scale bar = 50 μm.

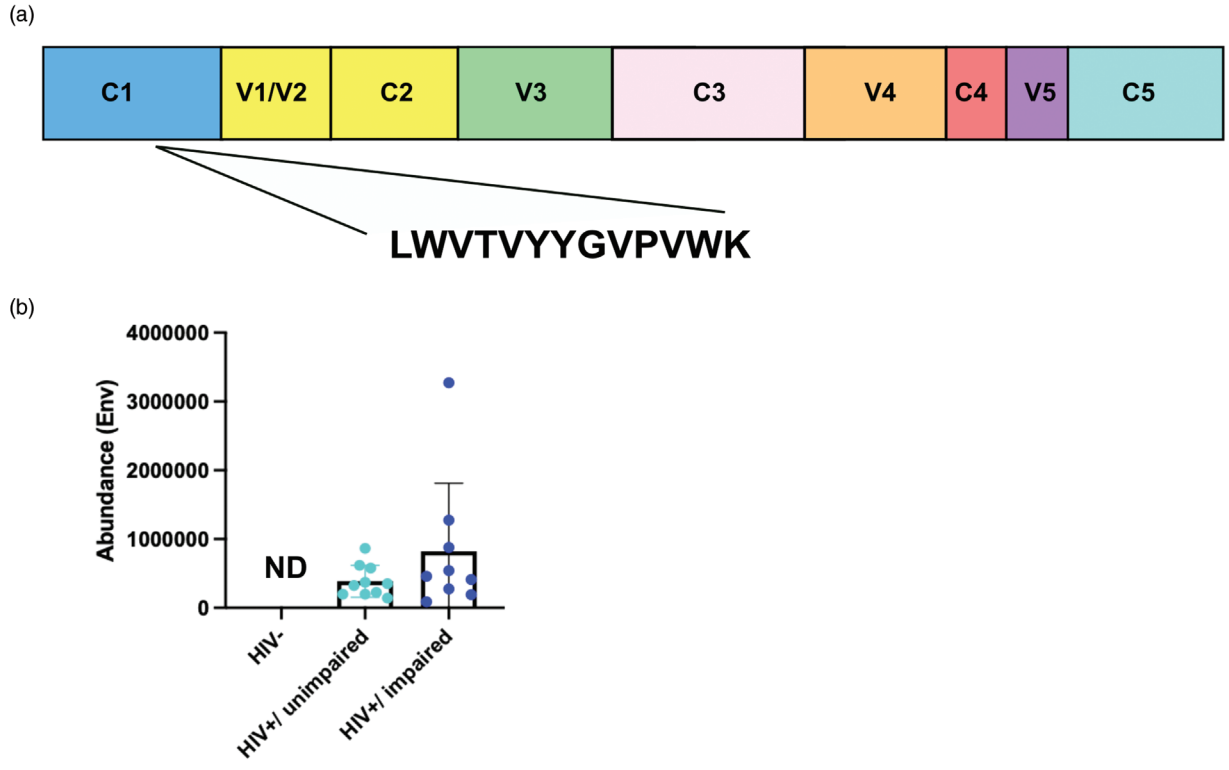

Fig. 4. Identification of gp120 peptide in cerebrospinal fluid samples. (a) The sequence of gp120 as shown in UniProt database from the accession numbers P03375 and P03377. C1–C5 are conserved regions of the protein, V1–V5 are variable regions. The gp120 peptide sequence detected in human cerebrospinal fluid (CSF) is shown in the C1 region. (b) Relative abundance of envelope gp120 in human CSF defined as the intensity of a specific peptide shown in panel (a) to that of total ions from a given sample. Gp120 was undetectable (ND) in all HIV-negative samples. All HIV-positive CSF samples are from people with HIV (PWH) treated with antiretroviral therapy (ART).

Gp120 is detectable in human cerebrospinal fluid

ART has substantially reduced HIVE and the severity of HAND, but not necessarily the incidence of HAND. To ascertain whether gp120 is in the CNS despite ART, we used a high-resolution NanoUPLC-MS/MS system to analyze 27 human CSF samples collected by the NIH-Neuro-HIV Research Consortium. Seven samples were from people without HIV, 10 from PWH without cognitive impairment, and 10 from PWH with impairment (Table 1). All HIV-positive CSF were from virally controlled study persons treated with ART. CSF proteins were analyzed by a nanoAcquity UPLC system with Orbitrap Fusion Lumos mass spectrometer in higher energy collisional dissociation mode. We were looking for a unique peptide sequence ^{33}LWVTVYYGVPVWK46 that belongs with high confidence (>95%) to the fragmentation of gp120 (Fig. 4a). This peptide sequence was validated using database (UniProt) matching and sequence alignment, and the accession numbers P03375 and P03377. In most HIV-positive samples, but not HIV-negative samples, we observed a double-charged peptide with an m/z value of 805.4390 (Supplementary Fig. 2, http://links.lww.com/QAD/D472), which contains the sequence described in Fig. 4a. All but one of the 20 HIV-positive samples contained the corresponding sequence of gp120, with a similar relative abundance (Fig. 4b). In contrast, no gp120 peptide fragments were detected in the HIV-negative CSF samples (Fig. 4b).

Discussion

Experimental studies have shown that gp120 binding to the co-receptors CXCR4 or CCR5 promotes neuronal degeneration [35,36]. Such binding could play a role in the mechanisms leading to pathogenesis of HAND and in the neuronal dysfunction, which occurs in PWH [37]. However, the presence of gp120 in the CNS in the ART era is still debatable. Using a dual approach, mRNA and protein analysis, we demonstrated that the env mRNA is expressed in the human CNS before ART, and that the gp120 protein is detectable in virally suppressed individuals.

Our data show that in the caudate nucleus and frontal cortex, both microglia and astrocytes express the env mRNA. In the caudate nucleus, the microglial and astrocytic expression of the env mRNA was widespread and varied among samples. We analyzed three sections per sample for env expression in astrocytes, and found that 10–50% of astrocytes in a randomly chosen field of view

expressed env mRNA. Similarly, we found that env expression in microglia varied widely across field of view. However, these samples are from PWH not treated with ART, thus, we would expect to find less env mRNA in PWH receiving ART. In the frontal cortex, the env mRNA in GFAP-positive cells appeared to be localized in agglomerates, and morphologically exhibited a feature of astrogliosis, characterized by large body. This is not a surprise because astrocytes have been reported by other investigators to become an HIV reservoir due to their ability to become persistently infected by HIV [5,6,33,34,38], and to transmit the virus to other cells [39]. Moreover, the postmortem brains used in our study are pathologically characterized by HIVE, which is often present in severe forms of HAND before ART. Thus, we cannot rule out that these astrocytes are a part of inflammatory responses. However, HIVE has become less common with ART and is not a necessary feature of HAND. Nevertheless, studies have shown the presence of HIV RNA and DNA in the brain of virally suppressed individuals [40,41]. Other studies have identified HIV RNA in the CSF [42], indicating that ART does not suppress the production of HIV protein transcripts. However, studies specifically investigating gp120 detection in the brain in the ART era are limited. This may be partly because of the genetic variability of gp120, which makes the use of a universal probe or marker challenging. Our data show that gp120 is present in almost all CSF samples (19 of 20) from PWH, even with ART. Our findings support the notions that ART may not be able to completely remove the production of viral proteins, and that the CNS becomes a sanctuary of HIV infection despite ART.

Although our postmortem immunohistology data show that both microglia and astrocytes express gp120 mRNA, the source of gp120 in the CSF remains unclear. Gp120, like other viral proteins, could be actively released from infected cells [43] and become available to the extracellular environment. In the CNS, these cells include resident immune cells, such as microglia, as well as nonresident macrophages [17,18]. In addition, perivascular astrocytes, which have been suggested to act as an HIV reservoir, can produce low levels of HIV virions when reactivated [34,44]. Gp120 can also be 'shed' from the virus during the initial phase of infection when the virus is incorporated into the host cells, but the envelope protein remains in the membrane [45]. Additionally, the likelihood of gp120 in the CSF originating from the periphery is implausible. The blood–CSF barrier is highly restrictive to the passage of molecules. Moreover, as these studies were performed on CSF of PWH with undetectable viral load due to ART, it is unlikely that the source of gp120 in these study samples is from the periphery.

HAND is most likely caused by multidimensional and complex immunopathological processes that are governed by viral proteins and host factors [46] to promote a neurotoxic environment. Our data show that gp120 is expressed in the CNS, suggesting that this protein can participate in the neurotoxic events that promote loss of synapses, as demonstrated in animal models of HAND (review in [37]). Although gp120 alone may not drive progressive neuropathology, this viral protein may be one contributor to neurotoxicity in the absence of viral replication in some PWH, but not others. Moreover, gp120 may amplify CNS damage, such as chronic inflammation, in HAND. A major caveat of gp120, as for other viral proteins, is whether the concentrations of gp120 found in the human CNS are sufficient enough to cause HAND. This protein can be degraded in the extracellular space, or endocytosed through low-density lipoproteins [25] or by a chemokine receptor-mediated mechanism [47]. In this study, we did not have enough samples to provide a correlation between levels of gp120 and cognitive impairment. Nevertheless, in a relatively small cohort, we found that most PWH positive for gp120 exhibit cognitive alterations, specifically 9 of 10 CSF samples of PWH with cognitive impairment were positive for gp120. In addition, although most of the samples in Table 1 represent an individual study person, one study person was assessed over time. This study person is represented by samples 13 and 27 (Table 1). This allowed us to assess gp120 expression longitudinally in the CSF of this study person, and we detected increased expression over time. For this study person, the increase in gp120 expression over time correlated with an increase in global-deficit score. Although our study focused on establishing the presence of gp120 in the ART era and validating detection methods, future studies are needed with a larger cohort to investigate whether gp120 levels correlate with cognitive impairment. Furthermore, the current study was limited by the availability of post mortem brains where successful HIV treatment is achieved. This provides avenues for future studies to apply these reliable detection methods in such contexts.

In conclusion, this study used IHC and mass spectrometry to evaluate gp120 expression in human tissue and CSF in the pre-ART and post-ART era. Our data suggest that the env protein is present in the CNS of PWH despite ART. However, we need additional studies of a larger cohort of PWH using ART to establish whether levels of gp120 in the CSF could be a biomarker for cognitive impairment. HAND pathogenesis under ART remains complex and our studies underscore the need to further explore gp120 as a therapeutic target to prevent cognitive impairment in PWH.

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Conflicts of interest

There are no conflicts of interest.

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