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Article

Alterations in Brain Cannabinoid Receptor Levels Are Associated with HIV-Associated Neurocognitive Disorders in the ART Era: Implications for Therapeutic Strategies Targeting the Endocannabinoid System

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Abstract: HIV-associated neurocognitive disorders (HAND) persist despite the advent of antiretroviral therapy (ART), suggesting underlying systemic and central nervous system (CNS) inflammatory mechanisms. The endogenous cannabinoid receptors 1 and 2 (CB_1 and CB_2) modulate inflammatory gene expression and play an important role in maintaining neuronal homeostasis. Cannabis use is disproportionately high among people with HIV (PWH) and may provide a neuroprotective effect for those on ART due to its anti-inflammatory properties. However, expression profiles of CB_1 and CB_2 in the brains of PWH on ART with HAND have not been reported. In this study, biochemical and immunohistochemical analyses were performed to determine CB_1 and CB_2 expression in brain specimens of HAND donors. Immunoblot revealed CB_1 and CB_2 were differentially expressed in frontal cortices from HAND brains compared to neurocognitively unimpaired (NUI) brains from PWH. CB_1 expression levels negatively correlated with memory and information processing speed. CB_1 was primarily localized to neuronal soma in HAND brains versus a more punctate distribution on neuronal processes of NUI brains. CB_1 expression was increased in cells with glial morphology and showed increased colocalization with an astroglial marker. These results suggest that targeting the endocannabinoid system may be a potential therapeutic strategy for HAND.

Keywords: cannabinoid receptor; inflammation; astrocytes; immunohistochemistry

1. Introduction

Human immunodeficiency virus (HIV) in the antiretroviral therapy (ART) era has transformed from a terminal illness to a chronic disease with life expectancy approaching that of seronegative patients [1]. However, even despite effective virologic suppression with ART, HIV-associated neurocognitive disorders (HAND) persist, affecting up to 50% of people with HIV (PWH) [2]. HIV is known to seed the brain within days of infection, and while ART has proven effective at suppressing viral loads and reducing progression to acquired immunodeficiency syndrome (AIDS), it does not eradicate central nervous system (CNS) viral reservoirs [3, 4]. Persistent low-level HIV replication, chronic inflammation, ART neurotoxicity, and aging comorbidities are thought to contribute to the neuropathogenesis of HAND [5-8]. Important avenues for future investigation will involve optimizing HIV therapy within the CNS.

The mechanisms driving HAND are likely multifactorial but common prospective etiologies include disruptions in neuroinflammatory signaling and mitochondrial function [9], both pathways that are modulated by the endocannabinoid system (ECS) [10-

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12]. Indeed, impaired mitochondrial fission and fusion, dysregulated autophagy, premature apoptosis, and altered calcium homeostasis have all been implicated in the pathogenesis of HAND [9]. Postmortem brain studies of HAND decedents reveal persistent astrogliosis, microgliosis, inflammatory cytokines expression, and altered mitochondrial architecture [13-15]. Importantly, recent evidence has shown that the endocannabinoid system regulates neuronal and glial function in rodent brains [16]. Animal studies implicate the endocannabinoid system in brain functions that are commonly altered in HAND patients, including learning and memory, executive function, and reinforcement behavior [16].

The ECS represents a promising therapeutic target for increasing resilience to HAND. Rates of cannabis (i.e., marijuana) use are disproportionately high in this population with approximately 77% of HIV-infected adults reporting lifetime marijuana use compared to 44.5% in uninfected adults [17-19]. Many patients report that cannabis offsets symptoms of HIV and ART side effects, including neuropathic pain, nausea, myopathy, lipodystrophy, and mood problems [20, 21]. Cannabis may have promising utility in treating various neurodegenerative disorders with underlying inflammatory processes, and the expression of cannabinoid receptors is associated with Alzheimer's disease [22], multiple sclerosis [23], Huntington's disease [24], and Down's syndrome[25]. The expression of cannabinoid receptors (CB_1 and CB_2) has been reported in HIV encephalitis [26], but the receptor expression in patients with a psychiatric diagnosis of HAND on ART remains unknown. A greater understanding of how cannabinoid receptor expression is associated with HAND is critical in evaluating the therapeutic potential of cannabinoid drugs, as well as their potential side effects.

In this study, CB_1 and CB_2 receptor expression in postmortem brain specimens were investigated from a well-characterized cohort of HAND decedents on ART. CB_1 and CB_2 levels in the frontal cortex were assessed by immunoblot, and then CB_1 cellular localization was investigated using immunohistochemical techniques. Lastly, CB_1 and CB_2 levels were correlated with clinical covariates.

2. Materials and Methods

2.1 Study population

Brain specimens from a total of 24 HIV+ donors were acquired from the National NeuroAIDS Tissue Consortium (NNTC) (Institutional Review Board [IRB] #080323) (Table 1). All studies were conducted in accordance with the code of ethics of the National Institutes of Health and the University of California, San Diego. Neuromedical and neuropsychological examinations were performed on each case within a median of 12 months prior to death. Exclusion criteria for subjects included a history of CNS opportunistic infections or diagnoses unrelated to HIV infection that might impact CNS functioning, such as neurologic, psychiatric, or metabolic disorders. The most common pathologies described were systemic cytomegalovirus (CMV), Kaposi sarcoma (HHV-8), and hepatic disease. Diagnosis of HAND was made according to a standardized evaluation as described below [27].

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Table 1. Clinical characteristics of study population. Abbreviations: NUI, Neurocognitively unimpaired; ANI, Asymptomatic neurocognitive impairment; MND, Minor neurocognitive dysfunction; HAD, HIV-associated dementia.

Neurocognitive diagnoses	Age	Plasma viral load	CD4 count	ART ever taken
NUI	45	41700	N/A	3TC
NUI	30	40	72	ATR
NUI	32	400	54	CBV/FTV/RTV/SQV/TRU
NUI	38	29282	663	3TC/ABC/ATV/DDI/KTA/RTV/TFV/ZDV
ANI	63	50	516	3TC/ATR/CBV/D4T/EFV/EPZ/IDV/NFV/TFV/TRU/ZDV/DRV/RTV/RPV/ATV
ANI	49	400	1	3TC/D4T/DDI/EFV/FTC/FTV/KTA/NFV/T20/TFV/TRU
ANI	46	750000	18	3TC/ABC/APV/D4T/EFV/NFV/ZDV/CBV/TZV
ANI	48	9501	80	3TC/D4T/DDC/DDI/EFV/HU/IDV/NFV/NVP/RTV/SQV/ZDV
ANI	43	5722	63	3TC/CBV/D4T/IDV/NFV/NVP/SQV
ANI	56	750000	70	3TC/D4T/DDI/EFV/NFV/NVP/ZDV/KTA/RTV/TFV/CBV/SQV/TRU/FPV
ANI	39	1013	402	3TC/D4T/IDV/TZV
ANI	48	50	480	ABC/ATV/DLV/DRV/MVC/RTV/TFV/TMC
MND	58	4064	14	ATR/ATV/DRV/FTC/KTA/RGV/RTV/TFV/TRU/TZV
MND	44	133166	7	CBV/KTA/TFV
MND	48	53556	77	CBV/EFV/NFV/ZDV
MND	60	50	119	3TC/EFV/HU/TFV
MND	43	50	69	3TC/ABC/D4T/DLV/EFV/IDV/NFV/ZDV/CBV/DDI
HAD	56	61223	24	3TC/ABC/ATV/D4T/DDI/EFV/EPZ/FTV/IDV/KTA/NFV/NVP/RTV/T20/3TC
HAD	54	400	336	ATV/CBV/FTC/KTA/NVP/RTV/TFV/TRU
HAD	59	400	32	3TC/DDC/CDLV/EFV/KTA/TFV/ZDV
HAD	36	6952	63	D4T/3TC/IDV/NVP/RTV/ABC/KTA/NFV/DDI
HAD	51	605555	34	TFV/FTC
HAD	56	1631	8	CBV/NVP
HAD	35	85510	3	ABC/CBV/D4T/DDI/IDV/NVP/RTV/SQV/ZDV/CBV

2.2 Neuromedical and neuropsychological evaluation

All participants underwent a comprehensive neuromedical assessment that included a detailed medical history and structured set of examinations for detecting lifetime and current diagnoses [27, 28]. For their baseline assessment, all subjects had venipuncture, cerebrospinal fluid (CSF), and urine samples collected. Clinical data (plasma viral load [VL], postmortem interval, CD4 count, and neuropsychological measures were obtained for the HIV+ donor cohorts.

Neuropsychological evaluation for HAND diagnosis was performed across seven neurocognitive domains including executive function, motor skill, processing speed, episodic memory, attention/working memory, language, and visual perception, as described by[27]. Raw test scores were transformed into normally-distributed T-scores adjusted for demographic variables, including age, education, gender, and race based on normative samples of HIV- participants and then averaged across all tests to obtain a global cognitive T-score and within domains to obtain cognitive domain-specific T-scores[29]. Functional impairments in everyday life were assessed using the Lawton and Brody Activities of Daily Living questionnaire[30] and Patient's Assessment of Own Functional Inventory (PAOFI)[31]. HAND classifications, i.e., asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and HIV-associated dementia (HAD), were assigned based on participant responses to the everyday functioning questionnaires and performances on the neuropsychological test battery according to established criteria[2].

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2.3 ImmunoBlot

Tissues from the white matter (GM) and gray matter WM of the frontal cortices dissected at autopsy from HAND and neurocognitively unimpaired (NUI) brains were homogenized and fractionated, as described by[32], using a buffer that promotes separation of membrane and cytosolic fractions (1.0 mmol/l HEPES (Gibco, cat. no. 15630–080), 5.0 mmol/l benzamidine, 2.0 mmol/l 2-mercaptoethanol (Gibco, cat. no. 21985), 3.0 mmol/l EDTA (Omni pur, cat. no. 4005), 0.5 mmol/l magnesium sulfate, 0.05% sodium azide; final pH8.8). Human brain tissue samples (0.1 g) were homogenized in 0.7 mL of fractionation buffer (1.0 mM HEPES, 5.0 mM Benzaidine, 2.0 mM b-mercaptoethanol, 3.0 mM EDTA, 0.5 mM magnesium sulfate, 0.05% sodium azide, pH 8.8) containing phosphatase (Millipore cat# 524624) and protease inhibitor (Millipore cat# 539131) cocktails. The tissue homogenate was centrifuged at 5000 x g for 5 min at room temperature, and the resulting supernatant was collected, placed in appropriate ultracentrifuge tubes, and centrifuged at 436,000 x g for 1 h at 44 °C in a TL-100 rotor (Beckman Coulter, Brea, CA). The supernatant was retained and represented the cytosolic fraction, and the pellets were resuspended in 0.2 mL of buffer and re-homogenized to obtain the membrane fraction.

After determination of the protein content of all samples by bicinchoninic acid assay (Thermo Fisher Scientific, cat. no. 23225), membrane fractions were resolved by SDS-PAGE and transferred onto PVDF membranes using the iBlot transfer system (Invitrogen, cat. no. IB24001) and NuPage transfer buffer (ThermoFisher Scientific, cat. no NP0006). The membranes were incubated for 1 h in 5% bovine serum albumin blocking solution and phosphate-buffered saline-tween 20 (PBST). Membranes were then incubated overnight at 4°C with primary antibodies against CB_1 (Abcam, cat. no. ab23703) and CB_2 (Abcam, cat. No. ab3561). Importantly, both of these antibodies were validated by the manufacturer for specificity in CB_1 and CB_2 knockout mice. Following visualization, blots were stripped and probed with a mouse monoclonal antibody against β-actin (ACTB; Sigma Aldrich, cat. no. A5441) diluted 1:2000 in blocking buffer as a loading control. All blots were washed in PBST, and then incubated with species-specific IgG conjugated to HRP (American Qualex, cat. no. A102P5) diluted 1:5000 in PBST and visualized with SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific, cat. no. 34096). Images were obtained, and semi-quantitative analysis was performed with the VersaDoc gel imaging system and Quantity One software (Bio-Rad).

2.4 Correlational Analyses between CB_1 and CB_2 protein levels and cognitive T-scores

We examined the relationship between global and domain-specific cognitive T-scores and CB_1 and CB_2 expression separately using Pearson's R correlations.

2.5 Immunohistochemistry and double immunofluorescence

Free-floating 40 μm thick vibratome sections of human brains were washed with PBS three times, pre-treated for 20 min in 3% H2O2, and blocked with 2.5% horse serum (Vector Laboratories, cat. no. S-2012) for 1 h at room temperature. Sections were incubated at 4°C overnight with the primary antibody, CB_1 (Abcam, cat. no. ab23703) diluted in PBS. Sections were then incubated in secondary antibody, Immpress HRP Anti-rabbit IgG (Vector, cat. no. MP-7401) for 30 min, followed by NovaRED peroxidase (HRP) substrate made with NovaRED Peroxidase (HRP) Substrate Kit as per manufacturer's instructions (Vector, cat. no. SK-4800). Control experiments consisted of incubation with secondary antibody only. Tissues were mounted on Superfrost plus slides (Fisherbrand, cat. no. 12-550-15) and coverslipped with cytoseal (Richard Allen Scientific, cat. no. 8310-16). Immunostained sections were imaged with a digital Olympus microscope. For each

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case (n=4 NUI and n=4 HAND) a total of 3 sections (10 images per section) were analyzed in order to quantify the average number of immunolabelled cells per field of view. CB_1+ cells of glial morphology and pyramidal neuronal bodies were counted manually. The specimens were blind coded and then broken after quantification Background levels were obtained in tissue sections immunostained in the absence of primary antibody. Unfortunately, due to technical difficulties with eliminating background signal, tissue sections were not amenable to immunohistochemistry with the CB_2 antibody.

Double immunolabeling studies were performed to determine the percent colocalization of CB_1 receptors with GFAP+ (astroglia) and MAP2+ (neurons) cells in frontal cortices, as described[13, 33]. For this purpose, vibratome sections of human brains were immunostained with antibodies against CB_1 with GFAP (Sigma Aldrich, cat. no. G3893) for astrocytes and MAP2 (Santa Cruz Biotechnologies, cat# sc- 32791) for neurons. Sections were then reacted with fluorescent secondary anti- bodies, goat anti mouse IgG 488 (Invitrogen, cat. no. A11011) and goat anti rabbit IgG 568 (Invitrogen, cat. no. A11036). Sections were mounted on Superfrost Plus slides and cover-slipped with vectashield (Vector, cat. no. 1000). Sections were imaged with a Zeiss 63× (N.A. 1.4) objective on an Axiovert 35 microscope (Zeiss) with an attached MRC1024 laser scanning confocal microscope system (BioRad, Hercules, CA). The percent colocalization was quantified using Image J and the SQUAASH method[34]. An examiner blinded to sample identification analyzed all immunostaining.

3. Results

3.1. CB_1 and CB_2 expression are increased in HAND brains on ART

To determine the expression levels of CB_1 and CB_2 in brains of HIV+ donors, we analyzed frontal lobe lysates generated from WM and GM from HAND cases as well as NUI cases (Table 1). Brain lysate membrane fractions were analyzed for CB_1, CB_2 and ACTB levels by immunoblot. In brain lysates from WM from HAND cases, CB_1 protein band intensity increased in MND and HAND compared to NUI and ANI (Fig. 1 A). The intensity of the band corresponding to CB_2 was similar in all groups (Fig. 1 A). Densitometry analyses of bands for CB_1 showed protein levels were significantly increased ~2-fold in MND and HAD when compared to NUI and ANI (Fig. 1 B). Densitometry analysis of the band corresponding to CB_2 revealed no significant difference between groups (Fig. 1 C). In brain lysates from GM from HAND cases, CB_1 protein band intensity increased in ANI, MND, and HAND compared to NUI (Fig. 1 D). The intensity of the band corresponding to CB_2 was less intense in ANI, MND, and HAND compared to NUI (Fig. 1 A). Densitometry analyses of bands for CB_1 showed protein levels were significantly increased ~1.7-, 1.9-, and 2-fold in ANI, MND, and HAD, respectively, when compared to NUI (Fig. 1 E). Densitometry analysis of the band corresponding to CB_2 revealed significant reduction (~40%, 60%, and ~50%, respectively) in ANI, MND, and HAD, respectively, when compared to NUI between groups (Fig. 1 F). These results suggest that CB_1 and CB_2 expression levels are differentially altered GM in brains of decedents that were diagnosed with HAND.

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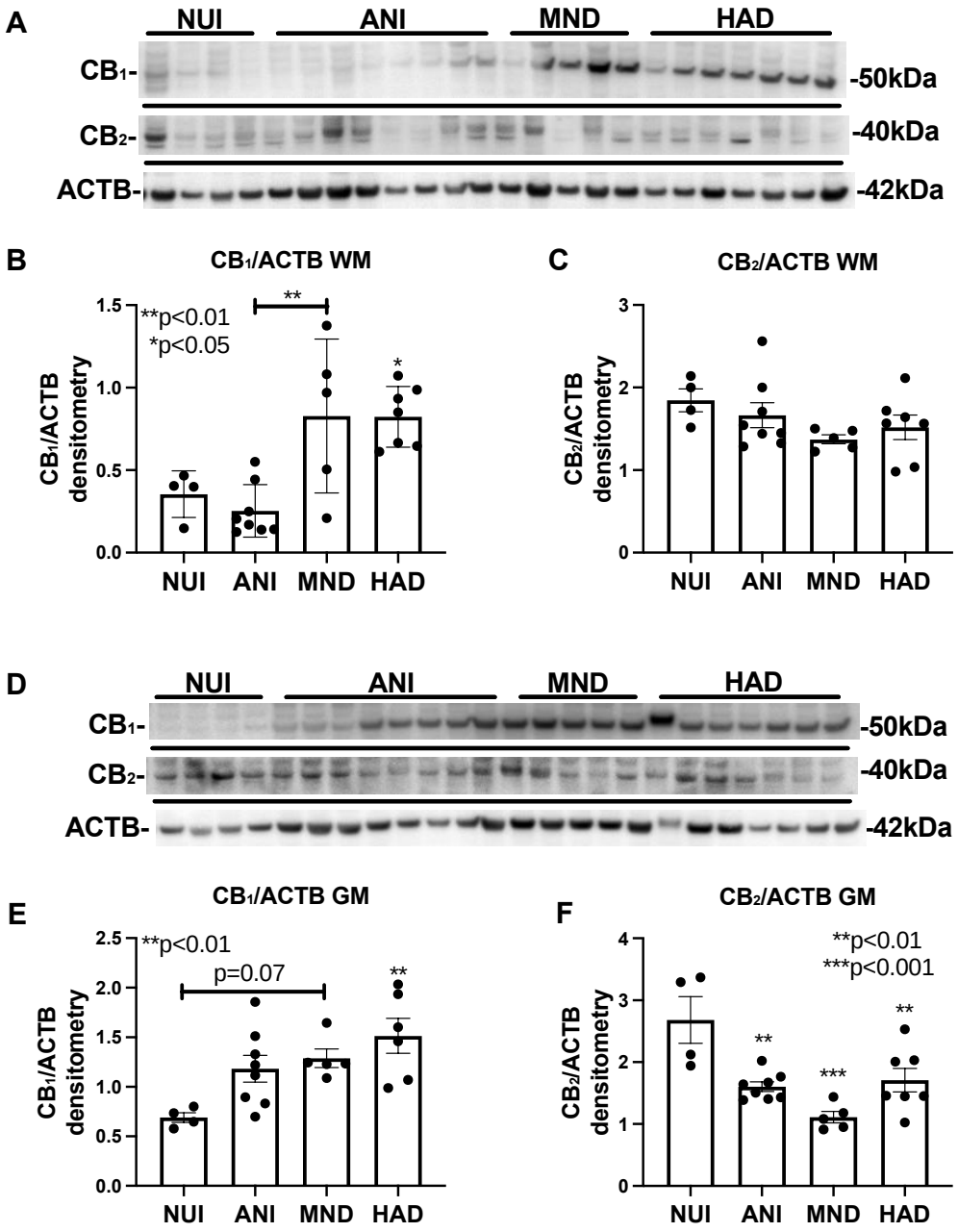

Figure 1: CB_1 and CB_2 expression are increased in HAND brains on ART. (A) Immunoblot of HAND donor WM frontal lobe lysates with antibodies specific for CB_1 and CB_2, and ACTB. (B and C) Quantification of normalized band intensity of CB_1 and CB_2 in WM stratified by HAND diagnosis. (D) Immunoblot of HAND donor GM frontal lobe lysates with antibodies specific for CB_1 and CB_2, and ACTB. (E and F) Quantification of normalized band intensity of CB_1 and CB_2 in GM stratified by HAND diagnosis. Statistical significance was determined by an one-way ANOVA followed by Tukey's multiple comparisons test. *, p<0.05, **, p<0.01, ***, p<0.001.

3.2. Elevated CB_1 expression in brains of PWH may indicate poorer cognitive function

As an exporatory analysis, as data were available, CB_1 and CB_2 expression levels in GM and WM were correlated with global and cognitive domain-specific T-scores. CB_2 expression levels in GM or WM did not relate to any cognitive outcome. Conversely, higher CB_1 expression levels in GM significantly related to poorer memory T-scores (R=-0.45, p=.04; Figure 3A) and higher CB_1 expression levels in WM significantly related to poorer speed of information processing (R=-0.49, p=.03; Figure 3B).

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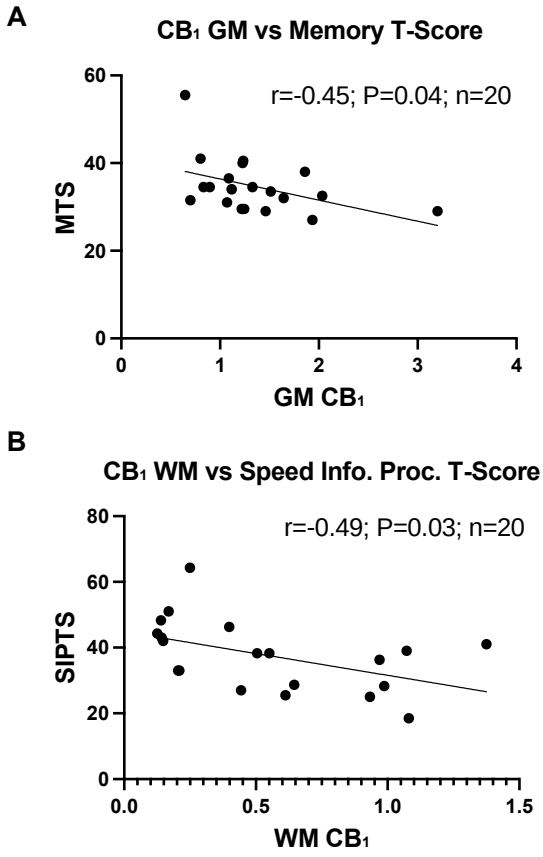

Figure 2: Greater CB_1 expression relates to worse cognitive function. (A) Correlation of CB_1 expression in GM with memory T-scores, R=-0.45, p=.04. (B) Correlation of CB_1 expression in WM detected by immunoblot with speed of information processing T-scores, R=-0.49, p=.03.

3.3 CB_1 expression and localization are altered in HAND brains on ART compared to NUI

To better understand the alterations in CB_1 levels in WM and GM of brains from HAND decedents, we performed immunolabelling for CB_1 in vibratome sections from the frontal cortex. First, we identified CB_1 expression in neurons in vibratome sections from the frontal cortex. The signal for CB_1 was clear and punctate throughout the neuronal processes in the GM in brains of HIV+ NUI decedents, however, CB_1 signal was very strong in soma of neurons in brains from HAND decedents (Fig. 2 A). Interestingly, the CB_1 dotting the neuronal processes throughout the GM was less common in brains from decedents diagnosed with HAND (Fig. 2 A). Next, to identify CB_1 co-localization with neurons, we double immunolabeled vibratome sections with antibodies against CB_1 and MAP2 and analyzed using confocal microscopy. In brains from decedents diagnosed as NUI, CB_1 stained strongly throughout the processes of MAP2+ cells (Fig. 2 B). However, the signal for CB_1 (red) was much more apparent in the soma of neurons in brains from decedents diagnosed with HAND when compared to CB_1 localization in brains from decedents diagnosed as NUI (Fig. 2 C). The quantification of the signal for CB_1 co-localizing with the signal for MAP2 was not significantly different in brains of decedents diagnosed as HAND when compared to CB_1 levels in brains of decedents diagnosed as NUI (Fig. 2 C). Next, we visualized CB_1 immunostaining in cells with glial morphology, which were apparent in WM but not GM. The signal for CB_1 was apparent in WM glial cells in brains of HIV+ NUI decedents. However, CB_1 signal was very strong in glial processes that extend from the soma more so in brains from HAND decedents than those from NUI decedents (Fig. 2 D). Strongly

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stained glia cells were present in WM but not in GM. Next, to identify CB_1+ astroglia, we double immunolabeled vibratome sections with antibodies against CB_1 and GFAP and visualized using confocal microscopy. In brains from decedents diagnosed as NUI, CB_1 stained lightly, with little colocalization with GFAP signal (Fig. 2 E). CB_1 stained more strongly in brains from decedents diagnosed with HAND (Fig. 2 E). Quantification of the signal for CB_1 co-localizing with the signal for GFAP increased by ~70% in brains of decedents diagnosed as HAND when compared to CB_1 levels in brans of decedents diagnosed as NUI (Fig. 2 F). These data suggest that astroglia increase expression of CB_1 during HAND, while CB_1 distribution in neurons may be abnormal in HAND, suggesting that astroglia may be an optimal therapeutic target using cannabinoids.

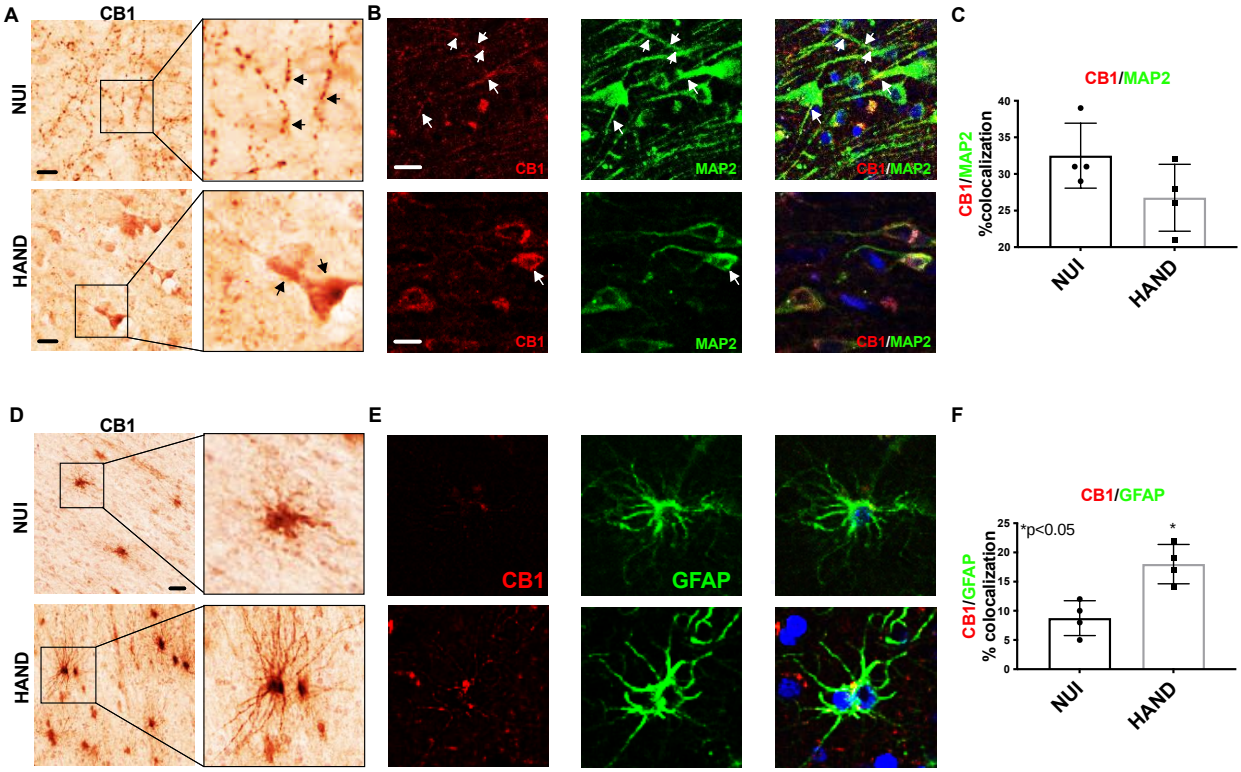

Figure 3: CB_1 expression and localization is altered in HAND brains on ART compared to NUI. (A) Immunostaining of CB_1 in the frontal cortices of NUI and HAND brain tissue. (B) Double-immunostaining of CB_1 and MAP2 in frontal cortices of HAND and NUI brain tissue. (C) CB_1 signal %colocalization of CB_1/MAP2. (D) Immunostaining of CB_1 in the frontal cortices of NUI and HAND brain tissue and the quantification of CB_1+ glia. (E) Double-immunostaining of CB_1 and GFAP in frontal cortices of HAND and NUI brain tissue. (F) Quantification of %colocalization of CB_1/GFAP. Statistical significance was determined by an unpaired t test. *, $p<0.05$.

4. Discussion

The current study provides evidence that the ECS is altered in brain tissues of HIV+ decedents that were diagnosed with HAND while on ART. This study is the first to analyze CB_1 and CB_2 receptor expression in HAND brains on ART. We identify pathological changes in CB_1 localization in neurons and astroglia that may reflect an attempt by brain cells to restore neuronal homeostasis. Correlational analyses between CB_1 levels and specific neurocognitive domains suggest that ECS changes associated with HIV and ART may be related to the development of HAND. These findings are consistent with previous studies that reported increases in CB_1 levels in PWH with HIV encephalitis and may implicate the ECS as a promising therapeutic target in PWH with HAND in the ART era[26] and in neuroinflammatory diseases in general[35].

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PWH use cannabis at higher rate than the general population, with 14-56% of PWH using cannabis compared to <10% in the general population[19, 36-40]. The higher rates of use in PWH may be due to cannabis ability to alleviate anxiety, depression, nausea, sleep disorders and other symptoms associated with HIV infection[20, 41]. While the evidence that cannabis effectively eliminates such symptoms in all patients is variable, our data suggest that the ECS is upregulated in response to HIV infection and ART, possibly as a compensatory mechanism to restore brain homeostasis. Future studies of how cannabis use affects the ECS in the brain in PWH are needed to better understand the therapeutic implications of cannabis.

Alterations in CB_1 and CB_2 expression levels may constitute a compensatory response to neuroinflammation that persists in PWH on ART despite low or undetectable viral loads [6, 7, 42]. In particular, increases in WM CB_1 may compensate for the observed decreases in WM CB_2 levels. Indeed, chronic inflammation in PWH on ART likely contributes to comorbidities such as HAND and depression. Several studies suggest that cannabinoids are anti-inflammatory and neuroprotective[10, 43-45]. Our recent studies showed that a cannabinoid receptor agonist, WIN55,212-2, may be neuroprotective by reducing neuroinflammatory gene expression in reactive astroglia, although we found that WIN55,212-2 was acting through peroxisome proliferator-activated receptors (PPAR) [13, 46]. Importantly, there is evidence that the phytocannabinoids isolated from cannabis, Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD), activate PPAR α and γ via signaling pathways that could be downstream of and also independent of CB_1 and CB_2 [47-49]. Other studies have shown that THC and CBD are neuroprotective in animal models for neurodegenerative diseases [45, 50]. Thus, THC and CBD may mimic endocannabinoid signaling mediated down modulation of neuroinflammation, a process that may be perturbed in PWH. These findings warrant further studies to delineate the mechanisms of cannabis mediated neuroprotection and the role of the ECS in these processes to better understand mechanisms of action underlying the therapeutic effects.

The neuronal pathology observed in HAND brains compared to NUI may reflect altered distribution of mitochondria throughout neuronal soma and processes. The CB_1 receptor has been shown to be located at mitochondria as well as on the plasma membrane of cells [51]. CB_1 has also been reported to be upregulated in neurodegenerative diseases [26]. Cannabinoid receptor agonists have shown promising therapeutic effects in multiple animal models for neurodegenerative diseases. Additionally, previous studies have shown that cannabinoid receptor agonists alter inflammatory gene expression and mitochondrial metabolic processes in multiple cell types[13, 20, 46, 47, 51-57]. Astroglia and neurons are highly involved in endocannabinoid signaling[54, 56, 58, 59]. Moreover, endocannabinoid signaling regulates neurotransmission and metabolism in and between the two brain cell types[12, 45, 49, 58-62]. These studies, for the first time, identify a shift in localization of CB_1 receptors on neurons from punctate distribution to localization to the soma of neurons, which may be associated with a shift in mitochondrial fission and fusion processes in neurons as we reported in HAND brains[32]. Indeed, our findings in NUI brains are consistent with previous findings that show a subset of CB_1 receptors dot the outer membranes of mitochondria where they alter function of the electron transport chain among other pathways [53, 55]. However, more studies are needed to confirm this hypothesis. In the least, the alterations in CB_1 and CB_2 expression and localization in HAND may indicate the ECS as a promising target for therapeutic intervention. This assertion is consistent with recent studies showing that PWH that use cannabis may demonstrate improved cognitive function compared to PWH not using cannabis[44, 63]. Future studies are needed to develop therapies aimed at astroglia to restore immunometabolic balance in the brain.

While important, relevant, and timely observations are presented, this study has several noteworthy limitations. A potential caveat in this study was the limited cohort size, which probably is not fully representative of the whole population of PWH. Future studies using brain specimens from a larger cohort that is demographically representative of PWH in regard to race, sex, age and other characteristics may more accurately

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elucidate the effects of HIV on CB_1 and CB_2 expression in the brain. Including HIV- controls in future analyses will be important to understand how HIV infection affects CB_1 and CB_2 expression independent of HAND status. Important specimen data on recency and frequency of exposure to cannabis and other drugs of abuse were not available for all participants and therefore limited the study to only correlations with HIV clinical data and neuropsychological measures of HAND. The analyses here are limited to only frontal cortex tissues, while CB_1 and CB_2 are expressed throughout the brain and in regions such as the hippocampus and striatum, which are implicated to be involved in HAND phenotypes. CB_1 and CB_2 are expressed in brain cell-types other than astroglia and neurons, including microglia and possibly oligodendroglia and it will be important to perform future studies that determine CB_1 and CB_2 levels in these cell types in specimens from PWH with HAND on ART. The ECS is composed of endocannabinoid-producing and -degrading enzymes as well as receptors aside from CB_1 and CB_2; none of which were examined in this investigation. CB_1 and CB_2 expression levels could not be related to markers of neuroinflammation or neurodegeneration as these data are currently unavailable; future studies are necessary to assess these correlations. Finally, the data presented here are observational and associative, lacking mechanistic links between HIV, ART, and the changes in CB_1 and CB_2. This work lays the foundation for future studies to identify HIV and ART-associated factors leading to alterations in CB_1 and CB_2 in the brain as well as a better understanding of the potential ameliorative role of cannabis in PWH.

5. Conclusions

Overall, this study identified alterations in CB_1 and CB_2 expression as potential mechanisms contributing to, or, alternatively, a consequence of the neuropathogenesis of HAND during the ART era. Importantly, these shifts in CB_1 and CB_2 expression may be dependent on cell-type and therefore could indicate that therapies designed to target these receptors or other ECS proteins in neurons or glial cells may be beneficial for HAND patients. These findings further support the recent interest in astroglia as mediators of neurodegenerative diseases and studies that show promising effects of cannabinoids in combatting such diseases [58, 64]. Future studies are needed to develop therapies aimed at astroglia and neurons to restore brain homeostasis.

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