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Cannabinoid Receptor Expression in HIV Encephalitis and HIV-associated Neuropathologic Comorbidities

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Abstract

Aims—Cannabinoids have been proposed for treating various neurodegenerative disorders and as adjunct therapy for HIV+ patients with neurologic sequelae. The expression of cannabinoid receptors (CB1 and CB2) has been reported in neurodegenerative diseases and in SIV encephalitis, yet the receptor expression in the CNS of HIV+ individuals is not known.

Methods—An anti-CB1 antibody and two anti-CB2 antibodies were employed for immunohistochemistry in the cerebral cortex and white matter of HIV encephalitis (HIVE) and HIV-associated comorbidities, as well as control brains (HIV− and HIV+).

Results—By quantitative image analysis, we observed that CB1 was increased in HIVE brains and those with comorbidities, while CB2 was significantly increased in the white matter of HIVE. Morphologically, CB1 was present in neurons, and both CB1 and CB2 were present in meningeal macrophages and subpial glia in all brains. In HIVE, CB1 was found in white matter microglia and perivascular cells, while CB2 was increased in microglia, astrocytes and perivascular macrophages. Double immunofluorescence with cell-specific markers and immunoblots on primary cultured microglia and astrocytes substantiated the glial localization of the cannabinoid receptors and specificity of the antibodies.

Conclusions—Our study indicates that cannabinoid receptor expression occurs in glia in HIVE brains, and this may have ramifications for the potential use of cannabinoid ligands in HIV-infected patients.

Keywords

cannabinoid receptor; human; inflammation; microglia; astrocytes; immunohistochemistry

INTRODUCTION

Cannabinoid receptor ligands and modifiers of endocannabinoids are under active investigation for potential use in numerous diseases ranging from neurodegenerative disease and neuropathic pain to cancer, as has been thoroughly reviewed (1–6). Neuroinflammatory and autoimmune conditions are also diseases where molecules of the endocannabinoid system (ECS), particularly the CB2 receptor, hold therapeutic promise (7;8). Recently, the expression of ECS proteins has been demonstrated in conditions such as Alzheimer's disease, multiple sclerosis, Huntington's disease and Down's syndrome (9–12). Furthermore, the expression of ECS proteins has been shown in the simian

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immunodeficiency virus (SIV) encephalitis, a macaque model of HIV encephalitis (HIVE) (13).

Despite the implementation of highly active anti-retroviral therapy (HAART), HIV-associated neurocognitive disorders (HAND) remain a problem in both westernized and developing nations (14–16). Adjunct therapies, including ECS modulators, have been proposed to downregulate inflammatory mediators that may contribute to HAND and to limit the neurological ramifications associated with HIV (7). Indeed, a recent study in a mouse model of neuroAIDS indicates that a CB2 agonist shows potential therapeutic value (17). An increased understanding of the expression of proteins of the ECS in human CNS would be useful in predicting how patients may respond to such proposed therapies and in elucidating the mechanisms of potential side effects for ECS-related compounds. It would also enhance our understanding of the role of ECS proteins in the pathogenesis of disease.

The ECS includes receptors such as cannabinoid receptor 1 (CB1), a constitutive central receptor mainly found on neurons, and cannabinoid receptor 2 (CB2), an inducible peripheral receptor mainly found on immune cells. Cannabinoids have also been shown to interact with non-cannabinoid receptors such as the TRP channel vanilloid receptor 1, GPR55, PPAR, which are under investigation (see (6) for review). In the CNS, endogenous cannabinoids such as anandamide and 2-arachidonoylglycerol (2-AG) among others can be produced by astrocytes and microglia, as well as neurons (18–20). Several enzymes, including fatty acid amide hydrolase (FAAH) 1 and 2, N-acylethanolamine acid amidase and monoacylglycerol lipase have been reported to hydrolyze endocannabinoids (21). Of these, FAAH is the most studied. In their investigation of the expression of ECS proteins in the SIVE model, Romero and colleagues observed that CB2 was upregulated in perivascular macrophages and microglial nodules and that FAAH was increased in perivascular astrocytes. In this report, we sought to investigate the expression of cannabinoid receptors and FAAH in the brains of HIV-infected individuals with encephalitis and HIV-related comorbidities.

MATERIALS AND METHODS

Patient Material

Formalin-fixed, paraffin-embedded sections from thirty-one brain specimens were acquired from several centers in the National NeuroAIDS Tissue Consortium (NNTC): twenty-two cases were from the Manhattan HIV-1 Brain Bank in New York, NY; five cases were from the National Neurological AIDS Bank in Los Angeles, CA; and four cases were from the Texas NeuroAIDS Research Center, in Galveston, TX. Brain regions included cerebral cortex (frontal, temporal or parietal) and associated white matter.

Table 1 summarizes the cases included in this study. Cases were divided into groups according to their HIV serostatus and the brain pathology: HIV-seronegative (HIV−), HIV-seropositive without encephalitis (HIV+), and HIV encephalitis (HIVE). Several HIV+/HIVE cases were also found to have CNS opportunistic infections, lymphoma or stroke and these were grouped together as comorbidities. The average age of individuals in the four groups was not significantly different. HIV− cases were 66.7% male but the percent of males in the HIV+ and HIVE groups was higher. 84% of all HIV+ patients had a history of combination antiretroviral therapy (cART) at some point in the past based on information from the NNTC database and retrospective chart analysis. Case information for a subset of MHBB cases has been published (22;23). Information on substance abuse obtained from patients' urine toxicology and retrospective chart analysis is provided in Table 1.

Immunohistochemistry for ECS Proteins

Brain sections were subjected to immunohistochemistry (IHC) for ECS proteins. IHC was performed for CB1, CB2 and FAAH. Staining for CB2 was performed with two different antibodies: one chosen because of its use in a previous report (13) and a second selected for comparison.

For IHC, brain sections were deparaffinized and subjected to antigen retrieval in 95 °C in modified citrate buffer at pH 6.1 (Target Retrieval Solution: DAKO, Carpinteria, CA) for twenty minutes. They were then treated with 3% H_2O_2, blocked with 5–10% normal horse serum in PBS for 30 minutes to one hour, incubated with the primary antibody and subjected to a polymer-based detection method (ImmPRESS) with diaminobenzidine (DAB) as the chromogen. All washes were done with phosphate-buffered saline. Single-stained slides were counterstained with hematoxylin, but double stained slides were not counterstained. For double-labeled IHC, IHC for ECS proteins was performed first and IHC for von Willebrand factor second as described (24). Table 2 summarizes the antibodies, reagents and methods for immunostaining.

Analysis of Single-stained Sections

Brain sections were analyzed for the distribution and location of positively-stained cells. Cells were identified as perivascular macrophages (PVC), astrocytes or microglia based on morphology and location. When parenchymal cells could not be positively identified, they are indicated as glia (see Table 1).

Immunofluorescence for ECS Proteins

Immunofluorescence for cannabinoid receptors and cellular markers was performed. Brain sections were deparaffinized and subjected to antigen retrieval in 95 °C sodium citrate buffer for twenty minutes. They were then blocked with 10% normal goat serum for 30 minutes to one hour. For most antibody combinations, double immunofluorescence was performed by combining the primaries into a cocktail and incubating the sections in the primary solution overnight at 4 °C. Extended incubations with the primary antibodies at 4 °C (two-three nights) were performed. Since the antibodies for mCB2 and HIV-1 p24 were both mouse clones, staining for these markers was performed sequentially. Immunofluorescence was completed with either the application of a primary and then a conjugated secondary (the two-step method, most antibodies), or via an avidin-biotin complex method (CB1 only). After the secondary step was completed, slides were not dehydrated, but were treated with 0.3% Sudan black (MP Biomedicals, LLC.; Solon, OH) in 70% ethanol for five minutes. This step is useful for quenching autofluorescence from lipofuscin (25). Slides were washed and mounted using Vectashield HardSet Mounting Medium containing 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstain (Vector Laboratories, Ltd.). Tonsil was used as the positive control for CB2 staining (26). Details regarding the antibodies, regents and methods used for immunofluorescence are provided in Table 2.

Digital imaging analysis for immunoreactivity of CB1 and CB2 in white matter

Digital image analysis was performed on samples with colorimetric staining and statistical analyses were performed as previously described (24;27). Briefly, 400X brightfield images were captured randomly using a Leica Leitz DMRB microscope and an Olympus DP12 digital camera. Twenty images were captured per case. After image capture, the immunoreactivity in the images was subjected to analysis using NIH ImageJ (28). Because of our interest in glial cells, and the presence of CB1 in neurons of all cases, images from white matter were selected for quantification of differential glial expression of cannabinoid receptors (27). A single threshold for background staining was used to process all images.

Analysis of the complete image was performed except for, in rare cases, where intravascular immunoreactivity or staining artifact was present. If this was the case, these items were removed and the remainder of the image was processed. The data are expressed as the percent of pixels in the image that are positive for DAB (brown color). Several cases (indicated in Table 1) that were immunostained had high levels of chromogen background or staining levels below the average threshold used and these cases were excluded from image analysis.

Digital imaging of immunofluorescence staining was performed at the Albert Einstein College of Medicine Analytic Imaging Facility (Bronx, NY) using an Olympus IX81 electronically motorized microscope (Olympus America, Inc.; Center Valley, PA) fitted with a Sensicam QE cooled CCD camera (Cooke Corporation, Auburn Hills, MI). The microscope was connected to a computer executing the IP Lab imaging software for Windows (BD Biosciences, Rockville, MD) through which images were captured. Images were processed (fluorophores merged) with NIH ImageJ.

Statistical analyses

Image analysis data were imported into GraphPad Prism Version 4.0 (GraphPad Software, La Jolla, CA) and the group data (HIV−, HIV+, HIVE and comorbidities) were compared using one-way analysis of variance (ANOVA) with Bonferroni multiple testing correction. To determine if CB1 staining was correlated with the level of CB2 staining, Spearman correlation was performed when data for both markers was available for a single case.

Analysis of cannabinoid receptor proteins by immunoblot

We assessed the cannabinoid receptor antibodies' ability to detect cellular protein by immunoblot. Highly enriched primary human fetal microglia and astrocyte cultures were prepared as previously described (29). Cell lysates were prepared with lysis buffer (10 mM Tris–HCl [pH 8.8], 50 mM NaCl, 0.5 mM Na_3VO_4, 30 mM $Na_4P_2O_7$, 50 mM NaF, 2 mM EDTA, 1% Triton X-100). Fifty μg of protein was separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membrane. Blots were blocked in a 1:1 ratio of 5% milk and 5% BSA in TTBS (Tris-buffered saline with 0.1% Tween-20). Primary antibodies were diluted in 5% BSA in TTBS at various concentrations (see Table 2) overnight at 4 °C. After incubation and washes in TTBS, the HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibody (Pierce Biotechnology, Rockford, IL) was applied at a concentration of 1:1000 at room temperature for two hours. Signals were developed using SuperSignal Pico or Femto enhanced chemiluminescence (Pierce Biotechnology). Blots were stripped with Restore PLUS Western Blot stripping buffer (Thermo Scientific, Rockford, IL) for 15 minutes. Blots were washed three times before re-blocking and re-probing with an additional antibody. Blots were probed for CB1, then re-probed for both CB2 antibodies, FAAH and β-actin as a loading control. See Table 2 for a summary of reagents, their sources and the concentrations used for immunoblotting.

RESULTS

Expression of cannabinoid receptors in white matter in control and HIVE cases (Figure 1)

HIV− and HIV+ cases both express small amounts of CB1 and CB2 in the white matter. The staining was present in white matter glia and perivascular macrophages was punctate and delicate (Figure 1A–F). Often, there were many puncta in a region, which may indicate that staining is present in cross-sections of cellular processes. More significant staining was observed in subpial glia (not shown) and meningeal macrophages (Figure 1G, H). Benito and colleagues used a polyclonal anti-CB2 antibody (13). In our hands, this CB2 antibody stained vessels and perivascular regions effectively (see Figure 1C, and also Figure 2), but

parenchymal glial staining was null to light. One exception was an HIVE case with confirmed lymphoma and a confounding bacterial infection. This case had high levels of glial staining in white matter (pCB2 < mCB2) (see below). In addition, some HIV+ cases had higher levels of mCB2 compared to HIV− cases. In addition to punctate staining, CB1 also showed cell staining, especially in HIVE or comorbidity cases. For example, serial sections of one HIVE case display the same perivascular cuff labeled with both CB1 (Figure 1I) and a macrophage marker, Iba1 (Figure 1J). CB2 labeling can be focal or diffuse. Serial sections from an HIVE case display mCB2 staining around a blood vessel (Figure 1K) that is positive for HIV-1 p24 in a stained serial section (Figure 1L). Based on morphology, cells staining with anti-CB1 are primarily macrophages and microglia, while those staining with the mCB2 are macrophages and microglia, multinucleated giant cells (K, left-hand inset) as well as reactive astrocytes (K, right-hand inset). Preliminary cellular analysis was performed on sections subjected to single immunohistochemistry for CB1 and mCB2. For single-stained cases, a description of cells positive for CB1 and mCB2 is provided in Table 1.

The expression of cannabinoid receptor proteins in the white matter of comorbidity cases (Figure 1)

Many individuals with HIV/AIDS are immunosuppressed and they present with one or more opportunistic infections or neoplasms (comorbidities). Several such cases (most with HIVE) were included in our analysis (Figure 1M–T). CB1 was very highly expressed in brains of comorbidity cases. In most cases, CB1 expression was focal and observed in areas of pathology (Figure 1M, N, Q, and S, respectively), but diffusely stained microglia were present in some cases (indicated in Table 1). In other cases, such as coccidiomycosis or PML, CB1 was more diffusely expressed in glia (Figure 1O, P, respectively). Macrophages in an infarct were positive for both CB1 and CB2 (see the serial section of the stroke case, Figure 1Q, R). Microglia in a nodule were more strongly stained with CB1 than with CB2, as observed in serial sections (Figure 1S, T).

Cellular expression of CB1, CB2 and FAAH based on location and morphology (Figure 2)

In previous reports, in human and non-human primate CNS, CB1 has been observed in neurons, activated macrophages, astrocytes, astroglioma cells and in oligodendrocytes, while CB2 has been observed in endothelial cells, astrocytes, activated and perivascular macrophages and microglia (9–11;13;30–33). Figure 2 confirms the presence of CB2 in many of those cell types based on morphology. In order to compare our findings with the work done in SIVE (13), we performed immunohistochemistry using the same antibodies, a polyclonal CB1 and CB2. CB1 was present in neurons of all cases, as shown in Figure 2A (an HIV− case is shown). CB2 was present in neurons in some cases, but was generally not observed in neuronal cells (B, C). FAAH was present in neurons (Figure 2D), but not in glial cells to any significant degree (not shown). With the pCB2, CB2 labeling was restricted to vessel-associated areas (perivascular cells, intravascular cells, endothelial cells, and vascular smooth muscle: Figure 2E, G, I–L). Significant parenchymal staining was not detected with the pCB2 in most cases of HIVE with the exception of a case with two comorbidities (a bacterial infection and lymphoma, Figure 2H). To confirm that pCB2 labeled vascular smooth muscle cells (Figure 2I, J), endothelial cells and perivascular cells, (Figure 2I– L), double-label IHC with von Willebrand Factor, an endothelial cell marker, was performed (J, L). Endothelial cells (K) and perivascular macrophages (L) are also shown to be positive for pCB2 in these double-labeled slides. Two cases with lymphoma did display significant staining with pCB2 (G, H).

Because the pCB2 did not display much parenchymal staining in our hands, we elected to use an additional antibody (mCB2) to confirm our findings. The mCB2 displays enhanced parenchymal immunoreactivity as compared to the pCB2 antibody as it is able to detect both

perivascular as well as parenchymal staining (Figure 2F). Perivascular and meningeal macrophages stained intensely with mCB2 (arrow in 2F, for example). Peri-neuronal microglia stained for mCB2 in gray matter in one case (M), as do microglia-like cells (N) and hypertrophic astrocytes (O). Staining in astrocytes was light and punctuate, with puncta often affiliated with the cell membrane.

Image analysis of CB1 and CB2 immunoreactivity (Figure 3)

Digitized photographs of CB1 and CB2 immunostaining were subjected to analysis with NIH ImageJ. The number of cases (n = 20) that were stained effectively for image analysis for CB2 was lower than that for CB1 (n = 27). The mCB2-stained sections were used for analysis because they had significant parenchymal staining as compared to the pCB2-stained sections.

The average % area stained for CB1 ranged from 0.03% (for HIV−) to 0.16% (for comorbidities). There was a graded increase in CB1 immunoreactivity among the HIV−, HIV+, HIVE and comorbidity groups (Figure 3A). The mean (± SEM) percent areas stained were compared among groups by one-way ANOVA and were found to be significantly different ($p < 0.001$). Pairwise comparisons with the Bonferroni multiple comparison test indicated that while the average percent area stained for CB1 in HIVE was significantly different from HIV− ($p < 0.01$), CB1 in comorbidity group was significantly different from both HIV− or HIV+ ($p < 0.001$).

The mean CB2 immunoreactivity is displayed in Figure 3B. For CB2 analysis, the lowest group average was 0.18% (for HIV−) and the highest group average was 0.58% (HIVE). The average percent area stained for mCB2 among all groups was significantly different ($p < 0.001$). The average mCB2 stained area for the HIVE group was significantly higher than HIV−, HIV+ or comorbidity groups ($p < 0.001$, $p < 000.1$ and $p < 0.01$, respectively).

While there are statistically significant differences among groups for both CB1 and CB2 immunoreactivity, analysis of individual cases within each group (Figure 3C and D) demonstrates a considerable variability from case to case. Within a group, some cases may display very low reactivity, yet others display very high immunoreactivity. Interestingly, despite having no active viral replication detectable by immunohistochemistry in the brain parenchyma (22), the HIV+ group had some individuals with very high levels of receptor staining.

Visibly, parenchymal CB1 staining was more intense than CB2 staining in serial sections of the same case (see Figure 1S and T, for example), but CB2 staining was stronger than CB1 in perivascular or meningeal regions (see Figures 1G, H, for example). The individual values for CB1 and CB2 were compared via Spearman correlation, but no correlation was observed (data not shown). In some cases, there was very high CB1 staining with no CB2 staining (M10013, for example) or very high CB2 staining with little CB1 staining (M10074, for example). The scales for CB1 and CB2 cannot be compared because the staining was qualitatively different in terms of background levels (polyclonal vs. monoclonal) and because different threshold levels were used.

Immunofluorescence confirms the presence of CB1 in microglia and CB2 in both microglia and astrocytes (Figures 4 and 5)

The image analysis confirmed an increase in both CB1 and CB2 in HIVE. We next wished to confirm the cell types expressing CB1 and CB2 in the white matter. To accomplish this, immunofluorescence was performed on six cases. We observed that CB1 is expressed primarily in microglia and perivascular macrophages in the white matter. Staining with the macrophage marker CD68 and CB1 were often overlapped (Figure 4A, B, C). Perivascular

cells (Figure 4A, B) and phagocytic parenchymal cells (C) were double-labeled. Only some HIV-1 p24+ cells expressed CB1 (Figure 4D). Although there were some identifiable CB1+ astrocytes visible on single-labeled sections, most GFAP+ astrocytes did not express CB1 (Figure 4E, F), providing additional confirmation that microglia and perivascular macrophages are the main cell types that express CB1 in HIVE cases. CB1 expression in neurons in the gray matter of an HIV− case (4G) and the white matter of an HIV− case and an HIV+ case (4H, I) are provided for comparison.

Microglial cells and perivascular macrophages express CB1, but they also express CB2. A macrophage/microglial marker Iba1 highlights a microglial nodule that is CB2+ (Figure 5A). Iba1+/CB2+ cells are shown in Figure 5F–H. Some Iba1-negative cells have astrocytic morphology (red cells in merge panel of A). A subset of GFAP+ cells expresses CB2 (Figure 5B, C) and in some regions, CB2+ astrocytes are abundant (Figure 5D). Similar to CB1, only some HIV-infected cells express CB2 (Figure 5E). To confirm that our immunofluorescence with CB2 was specific, a section of tonsil with CB2+ lymphoid follicle is included (Figure 5I).

Analysis of the cannabinoid receptor antibodies by immunoblot (Figure 6)

Our findings from this study indicate that CB1 is expressed mainly in microglia in HIVE and comorbidity cases, that CB2 is expressed in both microglia and astrocytes in HIVE, and that there are discrepancies in the immunolabeling with the mCB2 and pCB2. Therefore, we tested all three antibodies for their ability to detect cellular proteins by immunoblots. We used protein lysates from human fetal astrocytes (n = 6) and microglia (n = 6) and probed for CB1, mCB2, pCB2, FAAH and β-actin as a loading control (Figure 6). In microglia, CB1 and pCB2 showed a main band around 40 kD, corresponding to the native forms of these proteins (34). Other bands may indicate glycosylated forms of the receptors. The mCB2 antibody also detected a 40 kD protein in microglia, but a more prominent band was identified at 72 kD. Levels of CB1 detected in astrocytes were lower than those for microglia and a 40 kD band was only faintly visible. The pCB2 did not detect any specific bands in astrocytes (not shown) and the mCB2 also displayed either the 40 kD or the 72 kD band, similar to cultured microglia. The FAAH antibody did not detect any specific bands on our immunoblots (not shown).

DISCUSSION

Study of the expression of cannabinoid receptors in human CNS is of great interest because of the potential for cannabinoid receptor agonists or antagonists to act as therapeutics in various neuroinflammatory conditions. For example, CB1 inhibitors (e.g., Rimonabant) have been approved for treatment of obesity in Europe, but the psychological side effects prevented it from being approved in the U.S. and even caused its removal from the market (see (35) for review). Several studies have demonstrated that the peripheral immune CB2 receptor is expressed in activated microglia and macrophages in multiple human neurological disorders (8). These findings combined with the observation that CB2 receptor triggers anti-inflammatory signals in immune cells and microglia (7) have prompted the idea that selective CB2 agonists could be developed to treat neuroinflammatory diseases without eliciting the CB1-mediated psychoactive effects.

In this study, we employed detailed immunohistochemistry to determine the cannabinoid receptor expression in HIVE. Information on the cannabinoid system protein expression has been published in a number of human neurological conditions and in SIVE, but not in HIVE. In the current study, we found cannabinoid receptor expression in perivascular macrophages and subpial glia in all brains, including controls. This may indicate a role for these receptors in homeostatic surveillance. We also found that in the brains of HIVE, both CB1 and CB2

are upregulated in microglia and macrophages and that the CB2 is also increased in astrocytes. We believe the staining we observed with the CB1 antibody and CB2 was authentic for several reasons. First, the antibodies immunolabeled the positive controls (neurons for CB1 and a tonsil control for CB2), and the two polyclonal antibodies have been shown to be specific in tissue staining and immunoblotting based on pre-absorption studies (13;36). Second, the punctate nature of the staining evident in both controls and HIVE also corresponds to the known subcellular distribution of cannabinoid receptors in glial cells (37;38). Third, on immunoblotting of cultured human glial cells, the antibodies recognized molecular species corresponding to the cannabinoid receptor proteins reported from brain extracts and cell transfectants. The lack of correlation between CB1 and mCB2 staining is not unexpected because the two proteins are believed to be regulated differently (39;40). Our findings that both cannabinoid receptors are present in microglia and macrophages in human brain and that astrocytes might also express CB2 (see below) may help inform decisions on how to use newly developed cannabinoids as adjunct therapies in HIV patients.

Our finding of CB1 upregulation in macrophages and microglia in human neuroinflammatory disease is somewhat novel. Benito and colleagues also detected CB1 in macrophages associated with active plaques in multiple sclerosis (10). Our western blot analysis of cultured human fetal microglia demonstrated a highly consistent pattern (a single ~ 40 kD band) while astrocytes did not, supporting that CB1 might be a product of activated macrophages and microglia in vivo. Furthermore, in our ImageJ analysis, the upregulation of CB1 was progressive from HIV− to HIV+, HIVE and to the comorbidity group, though with considerable variations from case to case within the same group (Figure 3A and C). These results might suggest that upregulation of CB1 in macrophages and microglia in HIV-associated CNS conditions correlated with the amount of activated microglia and macrophages in these cases (see below).

With respect to the CB2 staining, our results in HIVE are somewhat different from those reported in SIVE (13). Benito and colleagues observed upregulated CB2 in microglia in SIVE using a polyclonal antibody against CB2 (pCB2). We find that the same pCB2 did not stain the brain parenchyma in most cases of HIVE, although vessel-associated cells (including intravascular leukocytes, vessels and some perivascular cells) were immunoreactive with pCB2. We attempted several staining methods for the pCB2 but could not attain significant parenchymal staining, even with the most sensitive micropolymer-based secondary method. The lack of microglial pCB2 staining is not due to the absence of activated microglia in these cases, as we have extensively documented activated microglia using several markers (CD68, CD45 and Iba1) in this and previous studies (22;24;41). Our immunoblot analysis with pCB2 demonstrated a ~ 40 kD band in all six cases of cultured fetal microglia but no bands in astrocytes. These results might indicate that the epitope recognized by the pCB2 might indeed be preferentially expressed by the peripheral immune cells.

Because of the lack of parenchymal staining with the pCB2, we have adopted another antibody (mCB2) for our study. The pCB2 was generated against a fusion protein including the first 33 amino acids of human cannabinoid receptor protein, while the mCB2 was generated against 293 HEK cell transfectants expressing the full-length human CB2. The mCB2 showed either a ~40 kD or ~ 72 kD band in both microglia and astrocyte culture lysates, most likely representing the native form and different glycosylated forms of CB2. Cannabinoid receptors have been shown to be post-translationally modified (such as by glycosylation) and alternatively spliced (34;42–47) and these alterations may account for the differences in epitopes for the two anti-CB2 antibodies.

Using the mCB2 antibody, we were able to detect CB2 expression in activated microglia, macrophages and astrocytes in HIVE. As with CB1, there was a great deal of case to case variation in the amount of mCB2 staining (Figure 3D). Quantitative analysis further demonstrated that the increase in mCB2 staining was found in HIVE. The increase of CB2 on macrophages and microglia in HIVE is in line with the study of SIVE by Benito et al (13). Although the Benito study did not state astrocyte reactivity for CB2, judging from the Figures (Figure 3C and D of reference (13) demonstrating typical perivascular astrocytic end feet forming the glia limitans), astrocytes were most likely a component of the glial CB2 in SIVE.

The within-group case to case variation in receptor expression is not unexpected. For example, CB2 gene polymorphisms associated with reduced levels of gene expression have been described in humans (48). Other uncontrollable variables that might lead to altered cannabinoid receptor expression include antiretroviral therapy and use of illicit substances, alcohol and tobacco (see Table 1 and data not shown). However, in the present study, the majority of HIV+ patients had a history of combination antiretrovial therapy, and therefore, cART does not appear to play a role in the expression of CB1/CB2.

Benito and colleagues displayed the FAAH protein in astrocytes in SIVE. Using the anti-rat FAAH IgG from a commercial source, we were unable to confirm that finding in HIVE. In our hands, the antibody stained neurons (known to produce FAAH), but not astrocytes. The FAAH antibody we used did not detect specific bands on western blot of human fetal astrocytes (data not shown).

Since our data in HIVE, as well as data from other human neurological diseases point to the conclusion that CB1 and CB2 receptor expression is upregulated in certain reactive glial cell populations, it was of interest for us to examine possible mechanisms behind such upregulation. Although complete maps of promoters and regulatory regions are not available for CNR1 and CNR2 (the CB1 and CB2 genes, respectively), numerous transcription factor binding sites may be present to regulate gene expression (44) and alternative transcriptional start sites have been observed in rodents (49). It is understood that immune cell transcription factors, such as STAT6, NF-κB, NFAT and AP-1, are involved in CB2 expression (40;50) and that cytokines such as IFNγ may be important in its upregulation (51;52). Transcriptional regulation of CB1 is also being actively studied (53).

In our preliminary exploration of glial cell cultures with several treatments such as HIV, a toll-like receptor ligand (LPS) or cytokines, we were unable to elicit consistent increases in cannabinoid mRNA or protein in cultured glial cells (data not shown). Although IL-1 with or without IFNγ did seem to cause some fluctuations in CB2 protein in astrocytes, there was no consistent pattern, and we are unable to see a change in gene expression in astrocytes by microarray (data not shown). At this time it is unclear what causes upregulation of the cannabinoid receptors in vivo. It is possible that combinations of factors including feedback by endocannabinoids or chronic exposure to multiple stimuli (such as nicotine or drugs of abuse) may be involved in ECS gene expression (see (54;55), for example).

Proposed functions of cannabinoid receptors in astrocytes and microglia include regulation of neurotransmitter release and reuptake (56), modulation of purinergic signaling (57;58), induction of anti-inflammatory genes ((59); see (2;8) for review), protection from apoptosis (60;61) and glial differentiation (62;63). Our work sheds light on the expression of ECS proteins in HIVE, but the exact roles of these proteins in HIVE remain to be determined. Further investigation into the causes and ramifications of ECS protein upregulation in human cells should increase our understanding of the functions of glial cannabinoid receptors and their potential as therapeutic targets in various CNS conditions.

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Non-standard abbreviations

2-AG	2-arachidonoylglycerol
ECS	endocannabinoid system
FAAH	fatty acid amide hydrolase
HAND	HIV-associated neurocognitive disorders
IHC	immunohistochemistry
NNTC	National NeuroAIDS Tissue Consortium
mCB2	monoclonal CB2 antibody
pCB2	polyclonal CB2 antibody

Reference List

1. Alexander A, Smith PF, Rosengren RJ. Cannabinoids in the treatment of cancer. Cancer Lett. 2009; 285:6–12. [PubMed: 19442435]
2. Ashton JC, Glass M. The Cannabinoid CB2 Receptor as a Target for Inflammation-Dependent Neurodegeneration. Curr Neuropharmacol. 2007; 5:73–80. [PubMed: 18615177]
3. Grant I, Cahn BR. Cannabis and endocannabinoid modulators: Therapeutic promises and challenges. Clin Neurosci Res. 2005; 5:185–199. [PubMed: 18806886]
4. Fowler CJ. Pharmacological properties and therapeutic possibilities for drugs acting upon endocannabinoid receptors. Curr Drug Targets CNS Neurol Disord. 2005; 4:685–696. [PubMed: 16375686]
5. Fride E. Endocannabinoids in the central nervous system--an overview. Prostaglandins Leukot Essent Fatty Acids. 2002; 66:221–233. [PubMed: 12052038]
6. Pertwee RG, Howlett AC, Abood ME, Alexander SP, Di MV, Elphick MR, Greasley PJ, Hansen HS, Kunos G, Mackie K, Mechoulam R, Ross RA. International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB and CB. Pharmacol Rev. 2010; 62:588–631. [PubMed: 21079038]
7. Cabral GA, Griffin-Thomas L. Cannabinoids as therapeutic agents for ablating neuroinflammatory disease. Endocr Metab Immune Disord Drug Targets. 2008; 8:159–172. [PubMed: 18782012]
8. Benito C, Tolon RM, Pazos MR, Nunez E, Castillo AI, Romero J. Cannabinoid CB2 receptors in human brain inflammation. Br J Pharmacol. 2008; 153:277–285. [PubMed: 17934510]
9. Benito C, Nunez E, Tolon RM, Carrier EJ, Rabano A, Hillard CJ, Romero J. Cannabinoid CB2 receptors and fatty acid amide hydrolase are selectively overexpressed in neuritic plaque-associated glia in Alzheimer's disease brains. J Neurosci. 2003; 23:11136–11141. [PubMed: 14657172]

10. Benito C, Romero JP, Tolon RM, Clemente D, Docagne F, Hillard CJ, Guaza C, Romero J. Cannabinoid CB1 and CB2 receptors and fatty acid amide hydrolase are specific markers of plaque cell subtypes in human multiple sclerosis. J Neurosci. 2007; 27:2396–2402. [PubMed: 17329437]
11. Nunez E, Benito C, Tolon RM, Hillard CJ, Griffin WS, Romero J. Glial expression of cannabinoid CB(2) receptors and fatty acid amide hydrolase are beta amyloid-linked events in Down's syndrome. Neuroscience. 2008; 151:104–110. [PubMed: 18068305]
12. Palazuelos J, Aguado T, Pazos MR, Julien B, Carrasco C, Resel E, Sagredo O, Benito C, Romero J, Azcoitia I, Fernandez-Ruiz J, Guzman M, Galve-Roperh I. Microglial CB2 cannabinoid receptors are neuroprotective in Huntington's disease excitotoxicity. Brain. 2009; 132:3152–3164. [PubMed: 19805493]
13. Benito C, Kim WK, Chavarria I, Hillard CJ, Mackie K, Tolon RM, Williams K, Romero J. A glial endogenous cannabinoid system is upregulated in the brains of macaques with simian immunodeficiency virus-induced encephalitis. J Neurosci. 2005; 25:2530–2536. [PubMed: 15758162]
14. Sacktor N. The epidemiology of human immunodeficiency virus-associated neurological disease in the era of highly active antiretroviral therapy. J Neurovirol. 2002; 8 (Suppl 2):115–121. [PubMed: 12491162]
15. Wong MH, Robertson K, Nakasujja N, Skolasky R, Musisi S, Katabira E, McArthur JC, Ronald A, Sacktor N. Frequency of and risk factors for HIV dementia in an HIV clinic in sub-Saharan Africa. Neurology. 2007; 68:350–355. [PubMed: 17261681]
16. Isezuo SA, Sani AZ, Ezunu E, Maiyaki S, Njoku CH, Obembe A. Clinical neuropathy in HIV/AIDS: an eight-year review of hospitalized patients in Sokoto, northwestern Nigeria. Trop Doct. 2009; 39:133–135. [PubMed: 19535744]
17. Gorantla S, Makarov E, Roy D, Finke-Dwyer J, Murrin LC, Gendelman HE, Poluektova L. Immunoregulation of a CB2 receptor agonist in a murine model of neuroAIDS. J Neuroimmune Pharmacol. 2010; 5:456–468. [PubMed: 20549374]
18. Walter L, Stella N. Endothelin-1 increases 2-arachidonoyl glycerol (2-AG) production in astrocytes. Glia. 2003; 44:85–90. [PubMed: 12951660]
19. Carrier EJ, Kearn CS, Barkmeier AJ, Breese NM, Yang W, Nithipatikom K, Pfister SL, Campbell WB, Hillard CJ. Cultured rat microglial cells synthesize the endocannabinoid 2-arachidonylglycerol, which increases proliferation via a CB2 receptor-dependent mechanism. Mol Pharmacol. 2004; 65:999–1007. [PubMed: 15044630]
20. Svizenska I, Dubovy P, Sulcova A. Cannabinoid receptors 1 and 2 (CB1 and CB2), their distribution, ligands and functional involvement in nervous system structures--a short review. Pharmacol Biochem Behav. 2008; 90:501–511. [PubMed: 18584858]
21. Fezza F, De SC, Amadio D, Maccarrone M. Fatty acid amide hydrolase: a gate-keeper of the endocannabinoid system. Subcell Biochem. 2008; 49:101–132. [PubMed: 18751909]
22. Cosenza MA, Zhao ML, Si Q, Lee SC. Human brain parenchymal microglia express CD14 and CD45 and are productively infected by HIV-1 in HIV-1 encephalitis. Brain Pathol. 2002; 12:442–455. [PubMed: 12408230]
23. Cosenza MA, Zhao ML, Shankar SL, Shafit-Zagardo B, Lee SC. Up-regulation of MAP2e-expressing oligodendrocytes in the white matter of patients with HIV-1 encephalitis. Neuropathol Appl Neurobiol. 2002; 28:480–488. [PubMed: 12445164]
24. Cosenza-Nashat M, Zhao ML, Suh HS, Morgan J, Natividad R, Morgello S, Lee SC. Expression of the translocator protein of 18 kDa by microglia, macrophages and astrocytes based on immunohistochemical localization in abnormal human brain. Neuropathol Appl Neurobiol. 2009; 35:306–328. [PubMed: 19077109]
25. Romijn HJ, van Uum JF, Breedijk I, Emmering J, Radu I, Pool CW. Double immunolabeling of neuropeptides in the human hypothalamus as analyzed by confocal laser scanning fluorescence microscopy. J Histochem Cytochem. 1999; 47:229–236. [PubMed: 9889258]
26. Carayon P, Marchand J, Dussossoy D, Derocq JM, Jbilo O, Bord A, Bouaboula M, Galiegue S, Mondiere P, Penarier G, Fur GL, Defrance T, Casellas P. Modulation and functional involvement

of CB2 peripheral cannabinoid receptors during B-cell differentiation. Blood. 1998; 92:3605–3615. [PubMed: 9808554]

27. Suh HS, Cosenza-Nashat M, Choi N, Zhao ML, Li JF, Pollard JW, Jirtle RL, Goldstein H, Lee SC. Insulin-Like Growth Factor 2 Receptor Is an IFNγ-Inducible Microglial Protein that Facilitates Intracellular HIV Replication: Implications for HIV-Induced Neurocognitive Disorders. Am J Pathol. 2010; 177:2446–2458. [PubMed: 20889566]
28. Abramoff MC, Magelhaes PJ, Ram SJ. Image processing with ImageJ. Biophotonics International. 2004; 11:36–42.
29. Lee SC, Liu W, Brosnan CF, Dickson DW. Characterization of primary human fetal dissociated central nervous system cultures with an emphasis on microglia. Lab Invest. 1992; 67:465–476. [PubMed: 1359193]
30. Calatozzolo C, Salmaggi A, Pollo B, Sciacca FL, Lorenzetti M, Franzini A, Boiardi A, Broggi G, Marras C. Expression of cannabinoid receptors and neurotrophins in human gliomas. Neurol Sci. 2007; 28:304–310. [PubMed: 18175076]
31. Schley M, Stander S, Kerner J, Vajkoczy P, Schupfer G, Dusch M, Schmelz M, Konrad C. Predominant CB2 receptor expression in endothelial cells of glioblastoma in humans. Brain Res Bull. 2009; 79:333–337. [PubMed: 19480992]
32. Jesus ML, Hostalot C, Garibi JM, Salles J, Meana JJ, Callado LF. Opposite changes in cannabinoid CB(1) and CB(2) receptor expression in human gliomas. Neurochem Int. 2010
33. Ellert-Miklaszewska A, Grajkowska W, Gabrusiewicz K, Kaminska B, Konarska L. Distinctive pattern of cannabinoid receptor type II (CB2) expression in adult and pediatric brain tumors. Brain Res. 2007; 1137:161–169. [PubMed: 17239827]
34. De Jesus ML, Salles J, Meana JJ, Callado LF. Characterization of CB1 cannabinoid receptor immunoreactivity in postmortem human brain homogenates. Neuroscience. 2006; 140:635–643. [PubMed: 16563642]
35. Bifulco M, Santoro A, Laezza C, Malfitano AM. Cannabinoid receptor CB1 antagonists state of the art and challenges. Vitam Horm. 2009; 81:159–189. [PubMed: 19647112]
36. Nunez E, Benito C, Pazos MR, Barbachano A, Fajardo O, Gonzalez S, Tolon RM, Romero J. Cannabinoid CB2 receptors are expressed by perivascular microglial cells in the human brain: an immunohistochemical study. Synapse. 2004; 53:208–213. [PubMed: 15266552]
37. Osborne KD, Lee W, Malarkey EB, Irving AJ, Parpura V. Dynamic imaging of cannabinoid receptor 1 vesicular trafficking in cultured astrocytes. ASN Neuro. 2009:1.
38. Brusco A, Tagliaferro PA, Saez T, Onaivi ES. Ultrastructural localization of neuronal brain CB2 cannabinoid receptors. Ann N Y Acad Sci. 2008; 1139:450–457. [PubMed: 18991892]
39. Samson MT, Small-Howard A, Shimoda LM, Koblan-Huberson M, Stokes AJ, Turner H. Differential roles of CB1 and CB2 cannabinoid receptors in mast cells. J Immunol. 2003; 170:4953–4962. [PubMed: 12734338]
40. Borner C, Hollt V, Kraus J. Activation of human T cells induces upregulation of cannabinoid receptor type 1 transcription. Neuroimmunomodulation. 2007; 14:281–286. [PubMed: 18287809]
41. Cosenza-Nashat MA, Kim MO, Zhao ML, Suh HS, Lee SC. CD45 isoform expression in microglia and inflammatory cells in HIV-1 encephalitis. Brain Pathol. 2006; 16:256–265. [PubMed: 17107594]
42. Song C, Howlett AC. Rat brain cannabinoid receptors are N-linked glycosylated proteins. Life Sci. 1995; 56:1983–1989. [PubMed: 7776822]
43. Liu QR, Pan CH, Hishimoto A, Li CY, Xi ZX, Llorente-Berzal A, Viveros MP, Ishiguro H, Arinami T, Onaivi ES, Uhl GR. Species differences in cannabinoid receptor 2 (CNR2 gene): identification of novel human and rodent CB2 isoforms, differential tissue expression and regulation by cannabinoid receptor ligands. Genes Brain Behav. 2009; 8:519–530. [PubMed: 19496827]
44. Zhang PW, Ishiguro H, Ohtsuki T, Hess J, Carillo F, Walther D, Onaivi ES, Arinami T, Uhl GR. Human cannabinoid receptor 1: 5' exons, candidate regulatory regions, polymorphisms, haplotypes and association with polysubstance abuse. Mol Psychiatry. 2004; 9:916–931. [PubMed: 15289816]

45. Onaivi ES, Ishiguro H, Gong JP, Patel S, Perchuk A, Meozzi PA, Myers L, Mora Z, Tagliaferro P, Gardner E, Brusco A, Akinshola BE, Liu QR, Hope B, Iwasaki S, Arinami T, Teasenfitz L, Uhl GR. Discovery of the presence and functional expression of cannabinoid CB2 receptors in brain. Ann N Y Acad Sci. 2006; 1074:514–536. [PubMed: 17105950]
46. Bermudez-Silva FJ, Suarez J, Baixeras E, Cobo N, Bautista D, Cuesta-Munoz AL, Fuentes E, Juan-Pico P, Castro MJ, Milman G, Mechoulam R, Nadal A, Rodriguez de FF. Presence of functional cannabinoid receptors in human endocrine pancreas. Diabetologia. 2008; 51:476–487. [PubMed: 18092149]
47. Matias I, Pochard P, Orlando P, Salzet M, Pestel J, Di MV. Presence and regulation of the endocannabinoid system in human dendritic cells. Eur J Biochem. 2002; 269:3771–3778. [PubMed: 12153574]
48. Ishiguro H, Horiuchi Y, Ishikawa M, Koga M, Imai K, Suzuki Y, Morikawa M, Inada T, Watanabe Y, Takahashi M, Someya T, Ujike H, Iwata N, Ozaki N, Onaivi ES, Kunugi H, Sasaki T, Itokawa M, Arai M, Niizato K, Iritani S, Naka I, Ohashi J, Kakita A, Takahashi H, Nawa H, Arinami T. Brain cannabinoid CB2 receptor in schizophrenia. Biol Psychiatry. 2010; 67:974–982. [PubMed: 19931854]
49. Sherwood TA, Nong L, Agudelo M, Newton C, Widen R, Klein TW. Identification of transcription start sites and preferential expression of select CB2 transcripts in mouse and human B lymphocytes. J Neuroimmune Pharmacol. 2009; 4:476–488. [PubMed: 19757078]
50. Borner C, Hollt V, Sebald W, Kraus J. Transcriptional regulation of the cannabinoid receptor type 1 gene in T cells by cannabinoids. J Leukoc Biol. 2007; 81:336–343. [PubMed: 17041005]
51. Maresz K, Carrier EJ, Ponomarev ED, Hillard CJ, Dittel BN. Modulation of the cannabinoid CB2 receptor in microglial cells in response to inflammatory stimuli. J Neurochem. 2005; 95:437–445. [PubMed: 16086683]
52. Racz I, Nadal X, Alferink J, Banos JE, Rehnelt J, Martin M, Pintado B, Gutierrez-Adan A, Sanguino E, Bellora N, Manzanares J, Zimmer A, Maldonado R. Interferon-gamma is a critical modulator of CB(2) cannabinoid receptor signaling during neuropathic pain. J Neurosci. 2008; 28:12136–12145. [PubMed: 19005078]
53. Mukhopadhyay B, Liu J, Osei-Hyiaman D, Godlewski G, Mukhopadhyay P, Wang L, Jeong WI, Gao B, Duester G, Mackie K, Kojima S, Kunos G. Transcriptional regulation of cannabinoid receptor-1 expression in the liver by retinoic acid acting via retinoic acid receptor-gamma. J Biol Chem. 2010; 285:19002–19011. [PubMed: 20410309]
54. Turu G, Varnai P, Gyombolai P, Szidonya L, Offertaler L, Bagdy G, Kunos G, Hunyady L. Paracrine transactivation of the CB1 cannabinoid receptor by AT1 angiotensin and other Gq/11 protein-coupled receptors. J Biol Chem. 2009; 284:16914–16921. [PubMed: 19357084]
55. Bosier B, Hermans E, Lambert D. Differential modulation of AP-1- and CRE-driven transcription by cannabinoid agonists emphasizes functional selectivity at the CB1 receptor. Br J Pharmacol. 2008; 155:24–33. [PubMed: 18536748]
56. Navarrete M, Araque A. Endocannabinoids mediate neuron-astrocyte communication. Neuron. 2008; 57:883–893. [PubMed: 18367089]
57. Witting A, Walter L, Wacker J, Moller T, Stella N. P2X7 receptors control 2-arachidonoylglycerol production by microglial cells. Proc Natl Acad Sci U S A. 2004; 101:3214–3219. [PubMed: 14976257]
58. Lane JR, Beukers MW, Mulder-Krieger T, Ijzerman AP. The endocannabinoid 2-arachidonylglycerol is a negative allosteric modulator of the human A3 adenosine receptor. Biochem Pharmacol. 2010; 79:48–56. [PubMed: 19665453]
59. Eljaschewitsch E, Witting A, Mawrin C, Lee T, Schmidt PM, Wolf S, Hoertnagl H, Raine CS, Schneider-Stock R, Nitsch R, Ullrich O. The endocannabinoid anandamide protects neurons during CNS inflammation by induction of MKP-1 in microglial cells. Neuron. 2006; 49:67–79. [PubMed: 16387640]
60. Cudaback E, Marrs W, Moeller T, Stella N. The expression level of CB1 and CB2 receptors determines their efficacy at inducing apoptosis in astrocytomas. PLoS One. 2010; 5:e8702. [PubMed: 20090845]

61. Gomez del PT, de Ceballos ML, Guzman M, Velasco G. Cannabinoids protect astrocytes from ceramide-induced apoptosis through the phosphatidylinositol 3-kinase/protein kinase B pathway. J Biol Chem. 2002; 277:36527–36533. [PubMed: 12133838]

62. Aguado T, Palazuelos J, Monory K, Stella N, Cravatt B, Lutz B, Marsicano G, Kokaia Z, Guzman M, Galve-Roperh I. The endocannabinoid system promotes astroglial differentiation by acting on neural progenitor cells. J Neurosci. 2006; 26:1551–1561. [PubMed: 16452678]

63. Galve-Roperh I, Aguado T, Rueda D, Velasco G, Guzman M. Endocannabinoids: a new family of lipid mediators involved in the regulation of neural cell development. Curr Pharm Des. 2006; 12:2319–2325. [PubMed: 16787257]

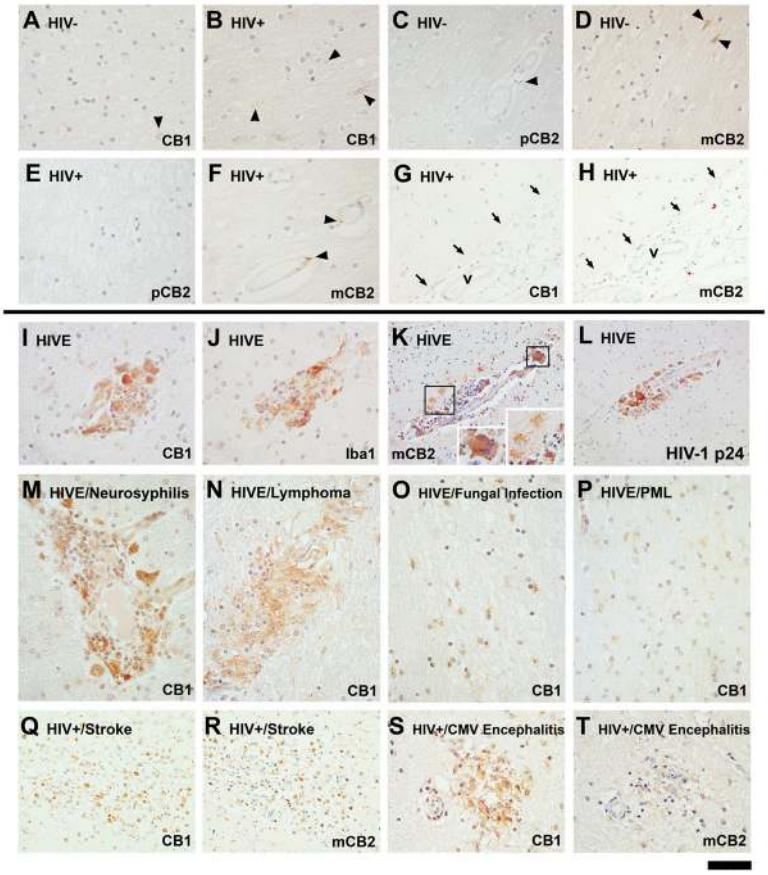

Figure 1.

Non-neuronal expression of cannabinoid receptors in control and HIVE brains. Three antibodies, a polyclonal anti-CB1 (CB1), a polyclonal anti-CB2 (pCB2) and a monoclonal anti-CB2 (mCB2) were used for immunohistochemistry in HIV−, HIV+ and HIVE cases with and without comorbidities, as described in the Materials and Methods. Regions shown are all from the white matter areas. A-H are from non-HIVE controls showing minimal parenchymal staining. Stained puncta are indicated with arrowheads. (A, B) Examples of minute levels of CB1 staining in HIV− and HIV+. (C, E) There is minimal pCB2 stain associated with vessels (arrowheads), while the parenchyma displays no staining. (D, F) In contrast, mCB2 detects staining in some glial cells, as well as perivascular macrophages (arrowheads). (G, H) In these serial sections from an HIV+ case stained with CB1 and mCB2, meningeal macrophages are positive for both, with CB2 being weaker than CB1. Arrows indicate the pial surface and "v" indicates the same subarachnoid vessel in serial sections. (I, J) Serial sections from the same HIVE case stained for CB1 and a macrophage marker, Iba1, demonstrate staining of perivascular macrophages. (K, L) Serial sections from the same HIVE case stain with mCB2 and anti-HIV-1 p24 show CB2 in perivascular macrophages, multinucleated giant cells (left inset) and adjacent hypertrophic astrocytes (right inset). HIV-1 p24 expression is limited to macrophages and multinucleated giant cells (L). (M – T) CB1 is present in cases with HIVE or HIV-associated comorbidities as indicated. In the cases with stroke (Q, R) or CMV (S, T), images captured from serial sections show that the mCB2 recognizes the same cell population (macrophages and microglia) as the CB1, though more weakly. The scale bar represents 50 μm in A-F, I, J, M–P and S–T and 100 μm in G, H, K, L, Q and R. DAB with light hematoxylin counterstain.

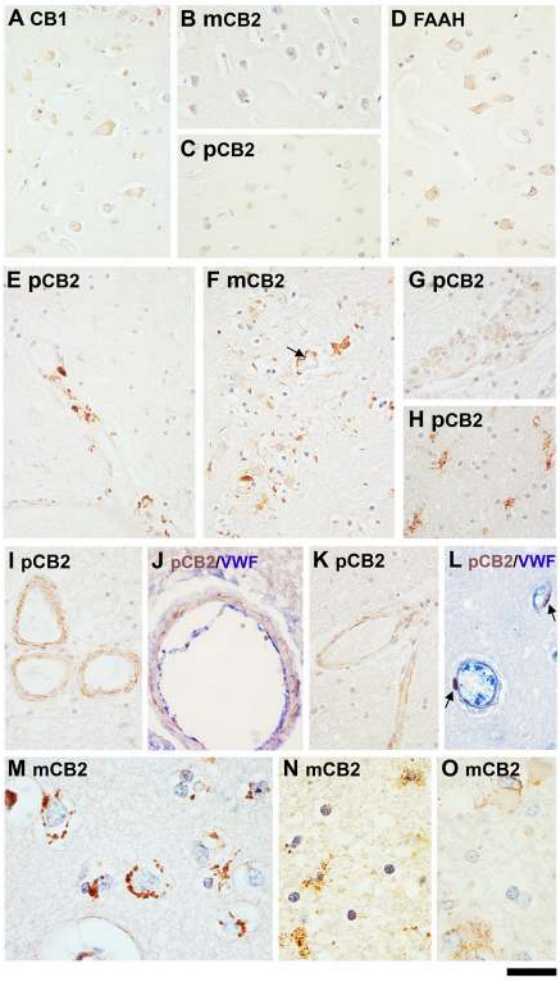

Figure 2.

Endocannabinoid system (ECS) protein immunohistochemistry in HIVE. (A–D) In the gray matter, CB1 and FAAH are present in neurons in all brain cases (HIV− cases are shown), but CB2 is not present in neurons in the vast majority of cases (see text). Glial ECS expression was detected primarily in the white matter. Images from similar white matter regions of the same HIVE case stained for pCB2 and mCB2 (E and F, respectively) show some differences in the detection of CB2 by the two antibodies. The pCB2 mainly detects vessel-associated cells (E), while the mCB2 detects both vessel-associated and parenchymal staining (F). Higher power depicting pCB2 stain in vascular smooth muscle (I, J), endothelial cells (K), and perivascular macrophages (L). (Double label for VWF, an endothelial marker, in blue: J and L). The pCB2 has none or little parenchymal staining in most cases. (G, H) However, the two cases with lymphoma show detectable levels of CB2 in perivascular regions and in glial cells, respectively. (M, N, O) Examples of parenchymal glial mCB2 stain in HIVE are shown in high power. Perineuronal microglia are strongly positive for CB2 (M), while parenchymal cells with microglial morphology (N) and hypertrophic astrocytes (O) are also positive. The scale bar represents 50 μm in A – L and 20 μm in M–O.

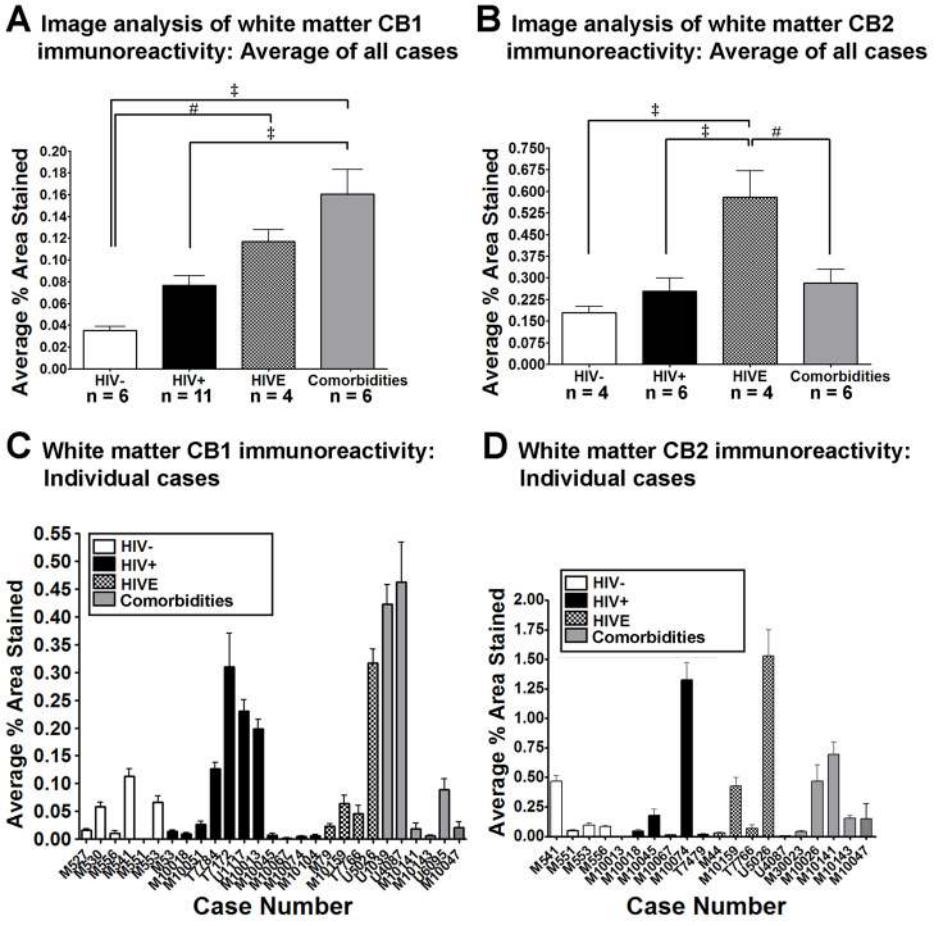

Figure 3.

Image analysis for CB1 and mCB2 immunoreactivity in HIVE and control cases with or without comorbidities. Sections were stained individually for CB1 and mCB2. Stained sections were photographed and twenty images per case were subjected to digital image analysis using NIH ImageJ software. Data are presented as the percent area stained (mean ± SEM) for CB1 (A, C) and for mCB2 (B, D). Data are displayed for four patient groups (A, B), as well as for individual cases (C, D). (A) The average CB1 staining in the HIVE group was significantly higher compared to HIV− control (# $p < 0.01$: One-way ANOVA with Bonferroni multiple comparison test). The comorbidities group showed further increase in CB1 stain compared to HIV− or HIV+ controls (‡ $p < 0.001$). (B) The averaged percent area stained for mCB2 immunoreactivity for the four groups is presented. Data show that CB2 expression in HIVE is significantly higher than in HIV− or HIV+ group (‡ $p < 0.001$). The comorbidities group shows lower CB2 average than HIVE (# $p < 0.01$). (C, D) The values for individual case percent areas are displayed. Cases from the MHBB (M), the Texas NeuroAIDS Research Center (T) and the National Neurological AIDS Bank at UCLA (U) are indicated. The data show variation in the amount of CB1 and mCB2 immunoreactivity in each patient group ranging from little or none to relatively high, but the variation does not appear to be due to different tissue origin. Note that cases for CB1 and CB2 analysis do not completely overlap (see Table 1 for details).

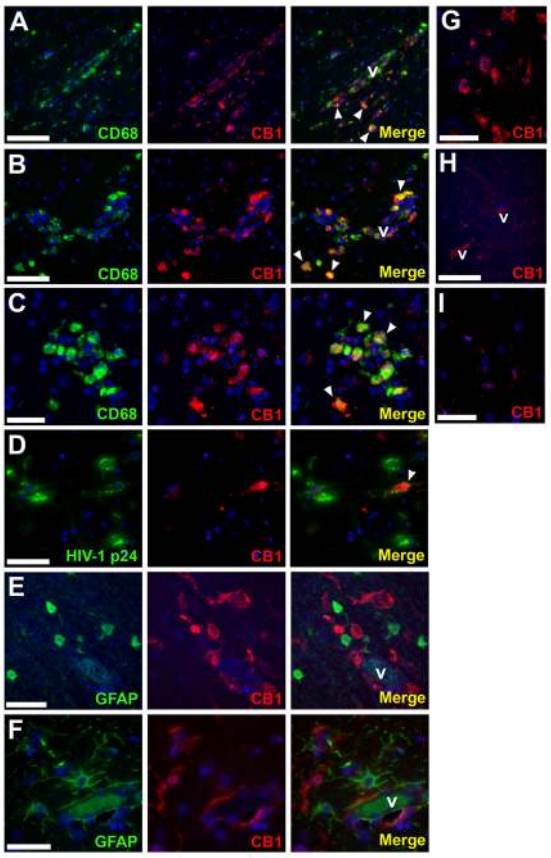

Figure 4.

Double immunofluorescence for CB1 and cell-specific markers show CB1 expression in microglia and macrophages in HIVE, in addition to neurons. Six cases were subjected to immunofluorescence for various cell markers. Images in A-F include representative images of HIVE white matter and consist of three panels each for the cell marker (CD68, HIV-1 p24, and GFAP), the cannabinoid receptor (CB1) and the image merge. Arrowheads indicate examples of double-positive cells. (A) A lower power image of a region with high CB1 staining shows the similarity of distribution of CD68, a macrophage marker, and CB1. "v" indicates a vessel. (B) CD68+ perivascular macrophages express CB1 (v = vessel). (C) A cluster of rounded parenchymal CD68+ cells in a microglial nodule are CB1+. (D) Some CB1+ cells express HIV-1 p24. (E, F) There are no CB1+ cells double labeled for the astrocyte marker GFAP (v = vessel). (G) All cases displayed CB1+ neurons in the gray matter (a representative HIV− case is shown). (H, I) The white matter in control cases displayed minimal CB1 (H: HIV− and I: HIV+, v = vessel). The scale bar represents 100 μm in A and H; 40 μm in B; 20 μm in C; 75 μm in D; 50 μm in E, G and I and 35 μm in F.

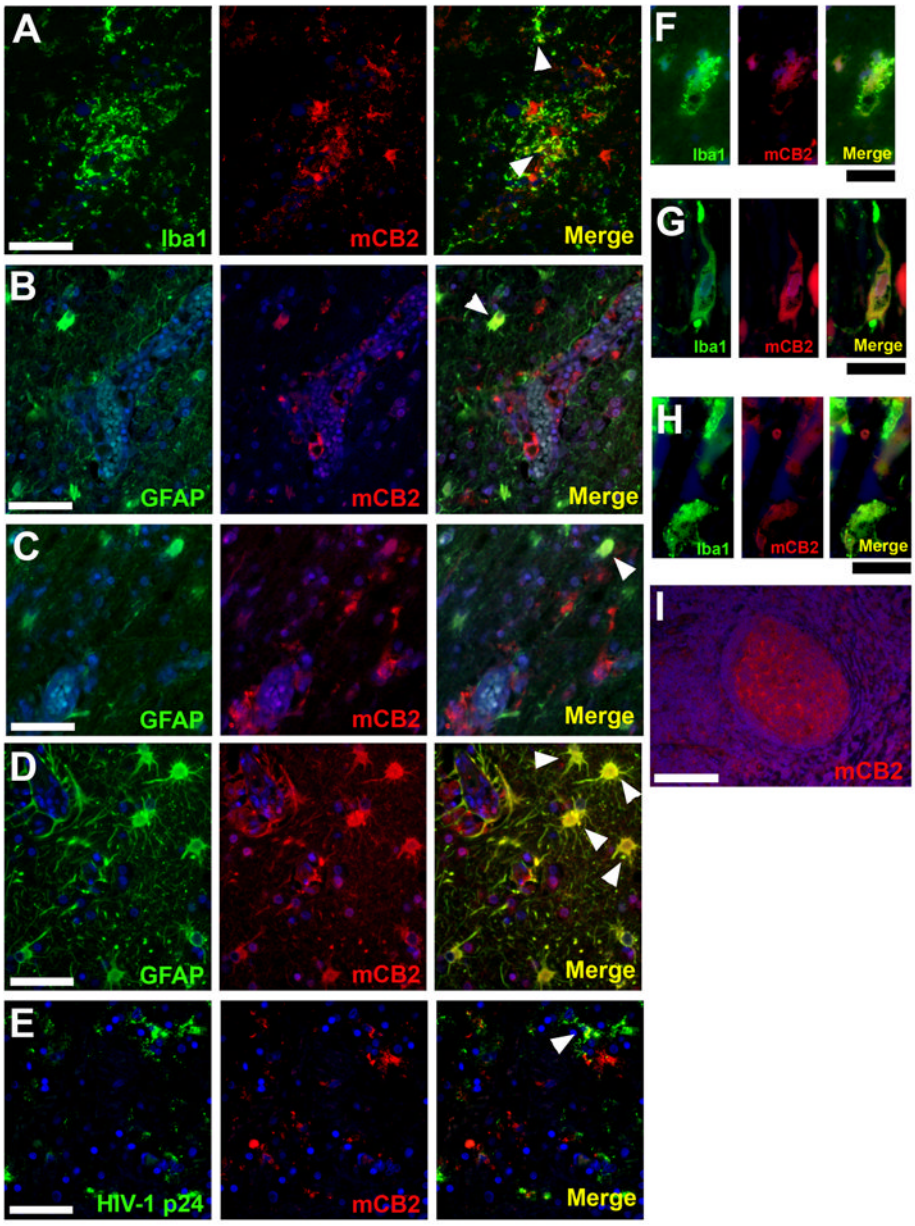

Figure 5.

Double immunofluorescence with cell-specific marker shows CB2 in microglia, macrophages and astrocytes of HIVE. Six cases were subjected to immunofluorescence for various cell markers. The images in A–H represent HIVE white matter and consist of three panels each for the cell marker, the cannabinoid receptor (mCB2) and the fluorophore merge. Examples of double-labeled cells are indicated with arrowheads. (A) A microglial nodule, visible with the macrophage marker Iba1, contains CB2+ cells. (B–C) Sections illustrate parenchymal, perivascular and intravascular CB2+ staining with a few cells in the parenchyma double labeled for GFAP. (D) Some regions in several cases had high CB2 expression in astrocytes. (E) Some HIV-1 p24+ cells also express CB2. (F–H) High power views of Iba1+/CB2+ parenchymal microglia. (I) The positive control for the CB2 antibody (human tonsil) shows a CB2+ lymphoid follicle. The scale bar represents 50 μm in A–E, 30 μm in F, G and H and 100 μm in I.

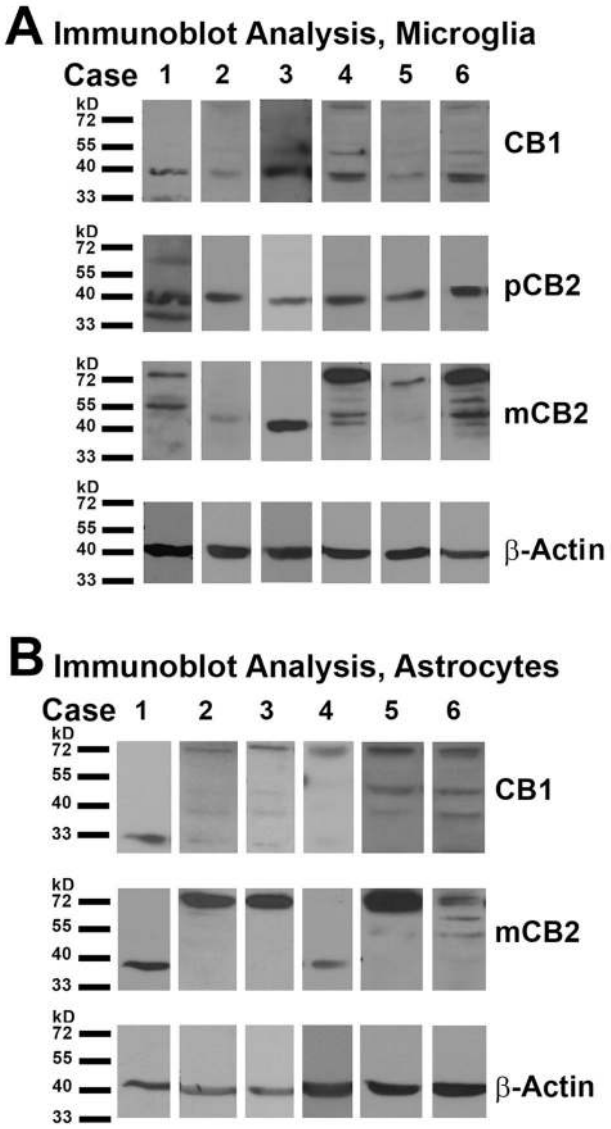

Figure 6.

Immunoblot analysis of the cannabinoid receptor antibodies using cell lysates of human fetal microglia and astrocytes. Six cases each of human fetal microglia (A) and astrocytes (B) were subjected to western blot analysis for cannabinoid receptors using the same three antibodies employed for tissue immunohistochemistry and fluorescence microscopy. β-actin was used as a protein loading control. (A) In cultured microglia, the CB1 antibody shows multiple bands with the 40 kD band being the dominant protein species in all; CB1 in its native form is reported to be ~ 37 kD. The pCB2 identifies a prominent single 40 kD band in all microglial cases. The native form of CB2 is reported to be ~ 39 kD. One case shows additional bands (Case 1). The mCB2 recognizes multiple species (40 kD, 55 kD and 72 kD band) variable from case to case. (B) In culture human fetal astrocytes, the CB1 antibody shows multiple bands, but the 40 kD band observed in microglia appears to be a minor protein. The pCB2 did not display immunoreactive bands in astrocytes (not shown). The mCB2 displays multiple bands, variable from case to case, similar to microglia. The FAAH antibody did not show specific bands in either microglia or astrocytes (not shown).

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Table 1

Human brain specimens used for CB1 and CB2 analyses.

Case number[1]	Status[3]	Age	Gender[3]	cART[3]	Substance Abuse	CB1+ PVC[3, 4]	Parenchymal CB1 stain[3, 4]	mCB2+ PVC[3,4]	Parenchymal CB2 Stain [3, 4]
M527	HIV−	40	m	n	None	y	Few scattered glia		NA
M530	HIV−	47	m	n	poly-drug use	y	Few scattered glia		NA
M556	HIV−	54	f	n	None	y	Few scattered glia	y	Diffuse glia
M541	HIV−	38	m	n	None	y	Few scattered glia	y	Diffuse astrocytes, diffuse microglia
M551	HIV−	30	f	n	None	y	No glial staining	y	Few scattered microglia
M553	HIV−	46	m	n	None	y	Few scattered glia	y	Few scattered astrocytes
	N = 6	Mean 42.5	66.7% m						
M53[5]	HIV+	41	m	n	Intravenous drug use (poly drug)	n	No glial staining		NA
M10018[5]	HIV+	39	m	y	Past poly-drug use	y	Diffuse astrocytes	y	Diffuse astrocytes
M10051[5]	HIV+	51	f	y	Methadone, past poly-drug use	y	No glial staining		NA
T7784	HIV+	41	m	y	Cannabis	y	Diffuse glia		NA
T7172	HIV+	44	m	y	None	y	Diffuse glia		NA
U1117	HIV+	55	f	y	None	y	Few scattered glia		NA
M10013	HIV+	33	m	y	Cannabis	y	Few scattered glia	y	Diffuse astrocytes
M10045	HIV+	31	f	y	None (Benzo-diazepine)	y	Few scattered glia	y	Diffuse microglia
M10067	HIV+	28	m	y	Cannabis	y	Diffuse glia	y	Few scattered glia
M10074	HIV+	41	m	y	Cannabis (past poly-drug use)	y	Diffuse glia	y	Diffuse microglia
M10104	HIV+	50	m	y	None	y	Few scattered and clustered glia	y	Diffuse astrocytes, few scattered microglia
T7479[2]	HIV+	41	m	y	Cannabis, past poly-drug use	y	Few scattered glia	y	Few scattered glia
	N = 12	Mean 41.3	73% m						
M44[2,5]	HIVE	32	m	n	Intravenous drug use (poly drug)	y	Diffuse glia	y	Scattered astrocytes
M79[2,5]	HIVE	45	m	n	Intravenous drug use (poly drug)	y	Microglial nodule		NA

Case number[1]	Status[3]	Age	Gender[3]	cART[3]	Substance Abuse	CB1+ PVC[3, 4]	Parenchymal CB1 stain[3, 4]	mCB2+ PVC[3,4]	Parenchymal CB2 Stain [3, 4]
M10159[5]	HIVE	47	f	y	Past poly-drug use	y	Few scattered astrocytes, microglial nodule	y	Diffuse astrocytes, Diffuse microglia
T7766	HIVE	38	m	y	None	y	Few scattered astrocytes, Diffuse microglia	y	Diffuse astrocytes, Diffuse microglia
U5026	HIVE	43	m	y	None	y	Few scattered astrocytes, microglial nodule	y	Diffuse microglia, few scattered astrocytes
	N = 5	Mean 41.0	80% m						
M10047	Comorbidity (stroke, old)	64	f	y	None	y	Few scattered glia; macrophages	y	Diffuse astrocytes, macrophages
U1039	Comorbidity (PML)	46	m	y	Cannabis	y	Few scattered astrocytes, diffuse microglia		NA
U4087	Comorbidity (bacterial infection, lymphoma)	36	m	y	None	y	Few scattered astrocytes, diffuse microglia	y	Diffuse astrocytes
M10141	Comorbidity (CMV encephalitis)	38	f	y	Cocaine	y	Few scattered astrocytes, microglial nodules	y	Diffuse astrocytes, microglial nodules
M10143	Comorbidity (CMV encephalitis)	47	f	y	Cannabis, (past cocaine)	y	Few scattered astrocytes, microglial nodules	y	Diffuse astrocytes, microglia nodules
U6095	Comorbidity (coccidiomycosis)	41	m	n	Cannabis	y	Diffuse microglia		NA
M30023[2, 5]	Comorbidity (lymphoma)	36	m	y	Cannabis (active poly-drug use)	y	Clustered astrocytes, microglial nodule	y	Diffuse astrocytes, diffuse microglia
M10026[2,5]	Comorbidity (neurosyphilis)	37	m	y	Past Alcohol	y	Few scattered astrocytes, diffuse microglia	y	Diffuse astrocytes, diffuse microglia
	N = 8	Mean 43.2	63% m						
Total	N = 31					N = 27		N = 20	

[1] Cases from the MHBB are indicated with M, from the TNRC are indicated with T and from the NNAB are indicated with U.

[2] Cases for which staining was excluded from ImageJ image analysis for one marker due to high background levels or non-detection at the threshold level with NIH ImageJ. When adequate staining was available, morphological analysis was performed without image analysis.

[3] Abbreviations stand for: m, male; f, female; PML, progressive multifocal leukoencephalopathy; CMV, cytomegalovirus; cART, combination antiretroviral therapy; NA, not available; PVC, perivascular macrophages; y, yes; n, no.

[4] Positive cells were identified on single labeled sections based on morphology and location. Glial cells were further identified as astrocytes or microglia based on morphology. In many control (HIV− and HIV+) cases, glia could not be further identified.

[5] Cases used and cited in prior studies (22;23)

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Table 2

List of antibodies and methods employed for immunohistochemistry, immunofluorescence and immunoblotting.

Antigen	Primary Antibody Source	Antibody Host	Primary Ab Concentration for IHC or Immune-fluorescence	IHC Method Employed	Immunofluorescence Method Employed	Primary Ab Concentration Used for Immunoblot
Cannabinoid Receptor 1 (CB1)	Originally, Affinity Bioreagents (Golden, CO) Currently, Thermo Scientific (Rockford, IL)	Rabbit polyclonal	1:300	ImmPress Anti-rabbit Peroxidase (overnight; Vector Laboratories, Inc.; Burlingame, CA)	Avidin-biotin complex method; Primary was followed by a biotinylated goat anti- rabbit IgG (Vector Laboratories, Inc.) and then a Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc.; West Grove, PA)	1:500
Cannabinoid Receptor 2 (pCB2)	Affinity Bioreagents, Thermo Scientific	Rabbit polyclonal	1:200	ImmPress Anti-rabbit Peroxidase (overnight)	Not Applicable (NA)	1:600
Cannabinoid Receptor 2 (mCB2)	R&D Systems (Min-neapolis, MN)	Mouse IgG2a	1:200	ImmPress Anti-mouse Peroxidase (one-day; Vector Laboratories, Inc.)	Primary was followed by a donkey anti-mouse Alexa fluor 546-conjugated secondary antibody (Invitrogen; Camarillo, CA)	1:500
Fatty Acid Amide Hydrolase	Cayman Chemical (Ann Arbor, MI)	Rabbit polyclonal	1:150	ImmPress Anti-rabbit Peroxidase (one-day)	NA	1:500
β-actin	Sigma (St. Louis, MO)	Mouse IgG2a	NA	NA	NA	1:500
CD68	DAKO (Carpinteria, CA)	Mouse IgG1	1:250	NA	Primary was followed by a goat anti-mouse Alexa fluor 488-conjugated secondary antibody (Invitrogen)	NA
Glial fibrillary acidic protein	Invitrogen	Rat monoclonal	1:100	NA	Primary was followed by a chicken anti-rat Alexa fluor 488-conjugated secondary antibody (Invitrogen)	NA
Iba1	Wako Pure Chemical Industries (Richmond, VA)	Rabbit polyclonal	1:500	Avidin-Biotin Complex Method with Vectastain kit, Alkaline Phosphatase (Vector Laboratories, Ltd.)	Primary was followed by a chicken anti-rabbit Alexa fluor 488-conjugated secondary antibody (Invitrogen)	NA
HIV-1 p24	DAKO	Mouse IgG1	1:10	Primary antibody followed by HRP-conjugated goat anti-mouse secondary antibody (Southern Biotechnology Associates, Inc., Birmingham, AL)	Primary was followed by a goat anti-mouse Alexa fluor 488-conjugated secondary antibody (Invitrogen)	NA