Cellular/Molecular

A Glial Endogenous Cannabinoid System Is Upregulated in the Brains of Macaques with Simian Immunodeficiency Virus-Induced Encephalitis

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Recent evidence supports the notion that the endocannabinoid system may play a crucial role in neuroinflammation. We explored the changes that some elements of this system exhibit in a macaque model of encephalitis induced by simian immunodeficiency virus. Our results show that profound alterations in the distribution of specific components of the endocannabinoid system occur as a consequence of the viral infection of the brain. Specifically, expression of cannabinoid receptors of the CB2 subtype was induced in the brains of infected animals, mainly in perivascular macrophages, microglial nodules, and T-lymphocytes, most likely of the CD8 subtype. In addition, the endogenous cannabinoid-degrading enzyme fatty acid amide hydrolase was overexpressed in perivascular astrocytes as well as in astrocytic processes reaching cellular infiltrates. Finally, the pattern of CB1 receptor expression was not modified in the brains of infected animals, mainly in perivascular macrophages, microglial nodules, and T-lymphocytes, most likely of the CD8 subtype. This could favor the CNS entry of infected monocytes.

Key words: neuroinflammation; viral encephalitis; gliosis; endocannabinoids; immunohistochemistry; macaques

Introduction

Cannabinoid CB1 and CB2 receptors, endogenous ligands (“endocannabinoids”), degradative and synthesizing enzymes for those ligands, and specific mechanisms for the termination of the biochemical signal are the basic elements of the endocannabinoid system (ECS) (Porter and Felder, 2001; Freund et al., 2003). It has been hypothesized that the ECS participates in several key processes that are related to, for instance, the control of motor activity, memory, reward, nociception, emesis, and endocrine functions (Portee, 2000). Furthermore, a prominent role for this system has been postulated in neuroprotection as well as in acute and chronic neurodegeneration (Grundy et al., 2001; Mechoulam et al., 2002).

However, the pathophysiological role(s) of the ECS in the CNS of higher mammals remains scarcely understood. Although subtle differences exist, it is currently accepted that basic aspects related to cannabinoid CB1 receptors and fatty acid amide hydrolase (FAAH) distributions in the human brain are in agreement with those observed in the rat (Tsou et al., 1998b; Freund et al., 2003). Cortical regions, basal ganglia structures, the hippocampus, and the cerebellar cortex exhibit high levels of expression of CB1 receptors and FAAH (Glass et al., 1997; Tsou et al., 1998a,b; Romero et al., 2002). It is also important to note that because of the massive postmortem generation of endocannabinoids (Felder et al., 1996), few data exist on their levels in the human CNS (Sarbitz et al., 2002). Finally, the selective expression of CB2 receptors in various immune cell types has been known since their discovery in 1992 (Munro et al., 1993). It is currently accepted that the ECS, mainly through CB2 receptors, participates in a wide variety of immune-related functions that range from antigen processing to macrophage migration or B-cell differentiation (Howlett et al., 2002). Very recently, our group has reported that CB2 receptors are present in both healthy brains (Nunez et al., 2004) and Alzheimer’s disease (AD) brains (Benito et al., 2003). Thus, we have described the presence of CB2 receptors in a discrete cellular subpopulation of brain macrophages, located on the external surface of blood vessels, in the brains of control patients (Nunez et al., 2004). These perivascular macrophages exhibit differential features from other types of microglia and play important roles in...
blood–brain barrier functionality (Williams et al., 2001b). This observation diverges from previous observations of rat and mouse brains (Lynn and Herkenham, 1994; Howlett et al., 2002) and suggests an additional role for the ECS in the human CNS. In addition, CB₂ receptor expression is induced in activated microglial cells associated with deposits of β-amyloid peptide in AD (Benito et al., 2003). Additional experimental data also found profound changes in FAAH distribution in AD tissue samples, because both FAAH protein and enzymatic activity are significantly increased in astrocytes surrounding β-amyloid-enriched plaques. Interestingly, no changes in CB₁ receptor patterns of distribution in AD samples were observed (Benito et al., 2003).

These observations suggest that the ECS undergoes dramatic changes in degenerative processes associated with neuroinflammation. This has led us to study the possible changes in specific elements of the ECS in an animal model of encephalitis.

Materials and Methods

Animals and sampling. Necropsy brain specimens from rhesus macaques (Macaca mulatta) with acquired immunodeficiency syndrome (AIDS) and simian immunodeficiency virus (SIV)-induced encephalitis (SIVE) (n = 4), SIV-infected animals with AIDS and without encephalitis (n = 2), and one normal, uninfected control were used in this study. Animals were inoculated with SIVmac251 (20 ng of SIV p27) by intravenous injection. All infected animals were administered the CD₈-depleting monoclonal antibody mT807 (5 mg/kg, i.v.) on days 6, 8, and 10 after infection. After the initial CD₈ depletion, there is a rebound of CD₈ T-cells. In animals with or without CD₈ depletion, nearly all T-cells in SIVE brains are of the CD₈ type (Kim et al., 2004). In this study, the main reason for using the CD₈ depletion paradigm was to induce more severe encephalitis with a higher incidence. When the animals developed AIDS, they were anesthetized with ketamine-HCl, killed by intravenous pentobarbital overdose, and exsanguinated. CNS tissues were collected in 10% neutral buffered formalin, embedded in paraffin, and cut into 5-μm-thick sections. The areas used in this study included frontal and parietal cortices. We chose these structures based on the well known changes that take place in these regions in SIVE, specifically in subcortical white matter.

Immunohistochemistry. The protocol used for the immunohistochemical staining is basically the same as described previously (Tsou et al., 1998a; Benito et al., 2003), with slight modifications. Briefly, tissue sections were deparaffinized and washed extensively in 50 mM potassium phosphate-buffered saline (KPBS) and endogenous peroxidase was blocked by incubation in peroxidase-blocking solution (Dako, Glostrup, Denmark) for 20 min at room temperature. To obtain a more efficient immunostaining, tissue sections were dehydrated and washed extensively in 50 mM potassium phosphate-buffered saline and incubated with biotinylated goat anti-rabbit HRP antibody (1:1000 dilution; Dako) for 24 h at 4°C. Afterwards, sections were incubated with anti-CB₁ antibody, followed by treatment with goat anti-rabbit antibody, and the complex was visualized using an ECL Western Blotting kit (Amersham Biosciences, Arlington Heights, IL). The specificity of the signal was confirmed by preincubation with the immobilizing peptide (1:900).

Results

Although no significant changes could be seen among control and SIVE animals with AIDS and without encephalitis, clear differences were evident in the pattern of expression of some elements of the ECS in the brains of SIVE animals. For this reason, joint data from control and SIVE animals without encephalitis are presented herein and compared with those from SIVE animals.
CB1 receptors

CB1 receptors were distributed homogeneously throughout the neuropil of the macaque cortex both in control and in SIVE animals (Fig. 1). In addition, cell bodies of pyramidal neurons were moderately stained, whereas no CB1 immunoreactivity was observed in white matter areas or in perivascular infiltrates (Fig. 1B). Finally, no significant changes were noticed in the pattern of expression and/or in the amount of CB1 protein (Fig. 2) between control and SIVE samples.

CB2 receptors

Although no significant staining was observed in tissue samples from control animals (Fig. 3A), an intense level of immunolabeling could be seen in SIVE animals. This signal was circumscribed to discrete cell populations. Specifically, perivascularly located cells exhibited the highest levels of staining (Fig. 3B), and remarkably some cells were also stained in white matter areas. These CB2-positive cells surround the external surface of almost all blood vessels, independent of the diameter of the vessels (Fig. 3B–D). In addition, CB2 immunostaining could be observed in cells located within cellular infiltrates (Fig. 3E,F). This signal corresponded to grouped cells and was limited to cellular infiltrates surrounding blood vessel walls, which is a well-characterized feature of SIVE brains.

To define the specific cell type(s) expressing CB2 receptors in SIVE animal tissue sections, double-labeling experiments were performed. To that end, two well known phenotypic markers were used: CD3 receptor (a marker for T-lymphocytes) and CD68 antigen (a marker for macrophages/microglia). Results obtained by this experimental approach allowed us to identify perivascular microglia and T-lymphocytes as CB2-expressing cells. Thus, CD68-positive cells within cellular infiltrates commonly found in perivascular areas of SIVE brains. Cells located both on incipient microglial nodules (arrows) and on well developed, mature infiltrates (dashed circle) show CB2 labeling. F. High-magnification image of an incipient infiltrate in the proximity of a blood vessel wall (asterisk) showing CB2 immunoreactivity (brown color). Scale bars: E, 800 μm; A, B, 400 μm; C, 200 μm; D, F, 100 μm.
T-cell subset (Kim et al., 2004), suggesting that these CD8-positive cells are also expressing CB₂ receptors.

**FAAH**

Cortical gray matter regions of control and SIVE macaques showed medium to intense immunoreactivity for FAAH (Fig. 5). Pyramidal neurons exhibited a strong signal in cell bodies. Interestingly, a clear perinuclear reinforcement could be noticed, whereas dendrites and axons were weakly stained; this resembles previous data obtained in human samples (data not shown) (Romero et al., 2002).

Dramatic changes were apparent in white matter areas of the cortex, an area known to suffer extensive astrogliosis in SIVE and human immunodeficiency virus (HIV)-induced encephalitis (HIVE). Thus, although no staining for FAAH was observed in white matter from control animals, strong immunoreactivity was detected in those with SIVE (Fig. 5B–F). This signal was present in astrocytes, as demonstrated by double staining with GFAP (Fig. 5E, F). Specifically, astrocytes surrounding perivascular cellular infiltrates showed high levels of FAAH immunostaining, in contrast to those surrounding vessels lacking pathological infiltrates, which showed only a moderate staining (Fig. 5C).

**Discussion**

We have studied the effects of brain infection by the simian immunodeficiency virus, a lentivirus that shares many similarities with HIV-1 (Lackner, 1994; Whetter et al., 1999; Nath et al., 2000). Brain infection by these viruses is thought to produce...
long-term damaging effects, including cognitive impairment and disruption of motor capabilities (Kolson et al., 1998; Kolson and Gonzalez-Scarano, 2000; Williams and Hickey, 2002). The SIVE model is considered a good experimental approach to the study of human disease because it mimics many of its principal hallmarks (Petry and Luke, 1997).

In humans, a subset of HIV-infected patients with dementia exhibit neuropathological changes, termed HIVE, in which microglial and astroglial cells play a pivotal role (Kolson and Gonzalez-Scarano, 2000; Kaul et al., 2001). HIVE is caused by a viral infection of the brain that targets perivascular brain macrophages (Williams et al., 2001a). Histopathology includes activated brain macrophages and astrocytes, inflammatory cuffs of monocyte/macrophages, some of which are infected, activated astrocytes, and inflammatory CD8 T-lymphocytes. Because neurons themselves are not infected directly, neuronal injury is thought to occur via indirect mechanisms by factors released from activated macrophages and astrocytes.

We report the existence of profound changes in the distribution pattern of cannabinoid CB1 and FAAH proteins in cortical regions of macaque brains affected by SIVE. These results fit well with those that we have observed previously in AD tissue samples (Benito et al., 2003) in that (1) they show dramatic changes in the distribution of several components of the ECS, (2) these changes affect mainly FAAH and CB2 receptors and not CB1, (3) the observed changes seem to be related to the inflammatory process characteristic of SIVE, and (4) a clear cellular selectivity exists for these changes, because FAAH is overexpressed in astrocytes, whereas CB2 receptors are expressed abundantly in perivascular microglial cells as well as in microglial nodules and infiltrated lymphocytes. These results allow us to postulate some elements of the ECS as markers for CNS inflammation and confirm our previous observations in AD, which show that an endocannabinoid glial system is upregulated in vivo under inflammatory conditions (Pazos et al., 2004). Furthermore, the similarities between FAAH and CB2 changes of expression in glial cells in SIVE and those observed in AD tissue samples suggest a common pattern of response, independent of the type of primary inflammatory insult.

The present data indicate that CB1 receptors remain unchanged in SIVE brains with respect to control brains and their presence is limited to neuronal elements of the cortex, as revealed by Western blotting and immunohistochemistry. This is in concordance with our previous data in human AD tissue samples (Benito et al., 2003) and seems to rule out a direct role for this subtype of cannabinoid receptors in the SIV-induced inflammatory reaction. It must be emphasized, however, that CB1 stimulation has been shown to confer neuroprotection and promote neuronal cell survival under different experimental paradigms (Guzman et al., 2002; Mechoulam et al., 2002), and thus a possible protective role for them in SIVE may be postulated. It is known that SIV and HIV do not infect neurons directly but instead productively infect microglial cells and macrophages, which then produce neurotoxic substances that ultimately kill neurons through a series of mechanisms including excitotoxicity, oxidative stress, and increases in Ca2+ levels (Kaul et al., 2001). All of these deleterious effects are known to be partially counteracted by CB1 activation (Van der Stelt et al., 2002). Interestingly, Maccarrone et al. (2004) have reported that a decrease in anandamide (a CB1 partial agonist) levels may be associated with the neurotoxicity caused by gp120 protein, one of the main virotoxins of HIV.

On the other hand, the results presented herein indicate that cannabinoid CB2 receptors may play a critical role in CNS inflammation. Their presence in perivascular microglial cells as well as in microglial nodules and in lymphocytes of the T type strongly suggests their involvement in several aspects of SIVE. First, we report that CB2 expression is induced in perivascular microglia as a consequence of viral infection, because control brains do not show CB2 immunoreactivity. This observation is in contrast with our previous data in control human brains that showed CB1-positive immunostaining in perivascular macrophages. As suggested in our previous work (Nunez et al., 2004), this could reflect an interspecies variation in the pattern of CB2 expression or an agonal/postmortem induction of CB2 expression in the human CNS. In any case, selective CB2 expression in this specific type of microglial cell suggests that this receptor may play a prominent role in the process of viral entry into the CNS, because perivascular microglia have been shown to be a cell type that is significantly involved in this process (Williams et al., 2001b). In this sense, it must also be emphasized that Croxford and Miller (2003) showed that viral load in the CNS is increased in the brains of mice inoculated with the Theiler virus, a murine model of multiple sclerosis, after treatment with the synthetic cannabinoid WIN55,212-2, a mixed CB1/CB2 agonist (Howlett et al., 2002).

Second, it is not surprising that CB2 receptors are present in microglial nodules. These cellular accumulations are one of the hallmarks of SIVE and HIVE. In addition, CB2 activation is currently considered a major factor in microglial function (Walter et al., 2003; Carrier et al., 2004) and, remarkably, has been shown to mediate the production of chemotactic substances for monocytes that are key factors in their recruitment into the brain. Klegeris et al. (2003) have reported that CB2 agonists decrease the production of proinflammatory substances by an activated human microglial cell line. Conversely, two groups have shown recently that CB2 activation also promotes microglial migration and proliferation (Walter et al., 2003; Carrier et al., 2004). Furthermore, CB2 activation enhances human macrophage-like and peripheral blood monocyte migration after CB2 activation (Deroq et al., 2000; Kishimoto et al., 2003).

Third, to our knowledge, this report is the first showing that CB2 receptors are present in peripheral cells that have infiltrated the CNS as a consequence of a pathological process. We demonstrate here that T-lymphocytes express this receptor in SIVE brains as a consequence of the viral CNS infection. Lymphocyte infiltration in tissues is a common feature in many forms of inflammation, and specifically in viral encephalitis, for which we and others have demonstrated a significant accumulation of CD8-positive T-lymphocytes that are likely SIV antigen specific (Kim et al., 2004). Although no conclusive data exist on the role of CB2 receptors in lymphocyte function (Grundy et al., 2001), these receptors are known to be expressed by peripheral T-lymphocytes, although in low levels. Their presence in these cells in the brains of SIVE animals is highly suggestive of a prominent role in CNS infiltration and could be a consequence of an upregulation process linked to brain invasion by these cells. In this sense, regulation of CB2 expression under different pathological conditions has been reported previously in other experimental paradigms (Sanchez et al., 2001; Jorda et al., 2003). Additional experiments should clarify this point.

Finally, a remarkable selectivity for FAAH expression in astrocytes was observed in SIVE samples. This feature matches well with previous data from AD human samples in which FAAH expression and activity were greatly enhanced in astrocytes located in the vicinity of neuritic plaques. Although tissue samples from control animals exhibited a wide distribution of FAAH in
principal neurons of the cortex (mainly pyramidal neurons), a massive expression of this enzyme in hypertrophied astrocytes was evident in SIVE samples. Furthermore, FAAH-positive astrocytes were found predominantly in perivascular regions and specifically in areas of cellular infiltration. It is currently thought that astrocytes become hypertrophied and hyperplastic in those brain regions in which an inflammatory process takes place and specifically that astrocytic processes densely infiltrate these areas as part of a concerted anti-inflammatory endogenous response (Wyss-Coray and Mucke, 2002). In our opinion, the presence of FAAH in these cells is highly suggestive of a direct involvement of FAAH in the inflammatory process linked to SIVE. In addition, astrocytes are known to play a regulatory role in HIV-1 encephalitis by dampening the overexpression of eicosanoids, platelet-activating factor, and tumor necrosis factor-α by activated HIV-1 monocytes (Minagar et al., 2002); FAAH overexpressed in glial cells could partially counteract some of these beneficial processes (Weber et al., 2004).

In summary, we report the existence of profound alterations in the pattern of expression of CB2 receptors and FAAH in the brain of macaques with SIVE. These changes are linked to the inflammatory process and suggest a prominent role for the ECS in glial activation under pathological conditions.

References


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