Cellular/Molecular

Cannabinoid CB_2 Receptors and Fatty Acid Amide Hydrolase Are Selectively Overexpressed in Neuritic Plaque-Associated Glia in Alzheimer's Disease Brains

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The endocannabinoid system is still poorly understood. Recently, the basic elements that constitute it, i.e., membrane receptors, endogenous ligands, and mechanisms for termination of the signaling process, have been partially characterized. There is a considerable lack of information, however, concerning the distribution, concentration, and function of those components in the human body, particularly during pathological events. We have studied the status of some of the components of the endocannabinoid system, fatty acid amide hydrolase and cannabinoid CB_1 and CB_2 receptors, in postmortem brains from patients with Alzheimer's disease. Using specific polyclonal antibodies, we have performed immunohistochemical analysis in hippocampus and entorhinal cortex sections from brains of Alzheimer's disease patients. Our results show that both fatty acid amide hydrolase and cannabinoid CB_2 receptors are abundantly and selectively expressed in neuritic plaque-associated astrocytes and microglia, respectively, whereas the expression of CB_1 receptors remains unchanged. In addition, the hydrolase activity seems to be elevated in the plaques and surrounding areas. Thus, some elements of the endocannabinoid system may be postulated as possible modulators of the inflammatory response associated with this neurodegenerative process and as possible targets for new therapeutic approaches.

Key words: **Alzheimer; astrocyte; astroglia; cannabinoids; immunoreactivity; microglia; neuropathology**

Introduction

The endocannabinoid system (ECS) performs many biologically important functions (Porter and Felder, 2001). The isolation and cloning of two different types of cannabinoid receptors, termed CB_1 and CB_2, partially unveiled the molecular mechanisms that mediate the well known effects of natural cannabinoids (for review, see Pertwee, 1997). Since then, putative endogenous ligands for those receptors have been isolated, and the mechanisms for termination of the biological signal have been identified. Thus, the ethanolamine of arachidonic acid [termed "anandamide" (AEA)] and 2-arachidonoyl-glycerol (2-AG) are the two main endogenous ligands (or "endocannabinoids") isolated so far (Mechoulam et al., 1998). On the other hand, a specific uptake mechanism and subsequent degradation of these compounds through the action of an amide hydrolase [fatty acid amide hydrolase (FAAH)] have been described (for review, see Giuffrida et al., 2001). Very recently, the possible existence of other subtypes of cannabinoid receptors, "non-CB_1 and non-CB_2," has been raised (Breivogel at al., 2001).

The distribution of the different elements of the ECS has been widely studied. Thus, it is currently accepted that although the cannabinoid CB_1 receptors are present in different tissues (predominantly of nervous origin), the cannabinoid CB_2 receptors are restricted to cell types related to the immune function (Grundy et al., 2001). CB_1 receptors are widely expressed in the CNS, being specially abundant in basal ganglia, hippocampus, cerebellum, and cortical structures (Herkenham et al., 1991; Mailleux and Vanderhaeghen, 1992a; Glass et al., 1997). This pattern of distribution matches well with the known effects of cannabinoids on motor and cognitive functions (Pertwee, 1997).

The presence of CB_2 receptors has been reported in spleen macrophages, tonsils, B cells and natural killer cells, monocytes, neutrophils, and T cells (Lynn and Herkenham, 1994; Galiegue et al., 1995), as well as in different cell lines (Grundy et al., 2001). Because of their presence in immune tissues, much work has been focused on the possible role of CB_2 receptors in mediating inflammatory responses (for review, see Parolaro et al., 2002); however, contradictory results have been obtained, partially because of the variety of experimental models and the doses of cannabinoids that were used (Grundy et al., 2001).

On the other hand, FAAH is considered to be one of the key elements in the regulation of the ECS function. It mediates the termination of the signal of AEA and possibly 2-AG (Deutsch et

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al., 2000, 2002), although it has been suggested that 2-AG is degraded *in vivo* primarily by the action of a monoglyceride lipase (Dinh et al., 2002). FAAH is an integral membrane protein and was originally cloned as the degrading enzyme of the sleep-inducing factor *cis*-9-octadecenoamide (oleamide) (Maurelli et al., 1995). Its cellular distribution in the rat brain has been studied by the use of different antibodies (Egertova et al., 1998; Tsou et al., 1998b) and by *in situ* hybridization (Thomas et al., 1997). Results were similar in these studies, showing that pyramidal cortical neurons, hippocampal pyramidal cells, and Purkinje cerebellar neurons exhibit the most intense immunostaining. FAAH displays a similar pattern of distribution in the human brain, being present in both neuronal and glial elements and showing a significant overlap with CB_1 receptors, mainly in areas related to motor control and memory (Romero et al., 2002).

Few data exist regarding the changes that the ECS may exhibit in normal or pathological aging. Autoradiographic studies in the past decade reported that CB_1 receptors are decreased in aged rats (Mailleux and Vanderhaeghen, 1992b; Romero et al., 1998) and that pathological conditions in the human affecting basal ganglia structures dramatically decrease the density of these receptors (Glass et al., 1993; Richfield and Herkenham, 1994). From these studies and from the known distribution of CB_1 and FAAH, the possible therapeutic interest of cannabinoid agonists and antagonists in motor diseases has been suggested (Fernandez-Ruiz et al., 2002). Thus, it seems of great importance to establish the status of the ECS in other pathological conditions affecting the human CNS, such as Alzheimer's Disease (AD).

Materials and Methods

Tissues. Postmortem brain tissues were obtained from four control brains (age range 64–75 years) and seven AD patients (age range 68–82 years) within a 12 hr postmortem interval. Control subjects had no background of neuropsychiatric disease, and the full neuropathological examination performed in every case on paraffin-embedded tissue excluded any significant pathological finding. On the other hand, all AD subjects met the CERAD (consortium to establish a registry for Alzheimer's disease) clinical and neuropathological criteria for the diagnosis of definite AD (Mirra et al., 1991). AD diagnosis was confirmed by Gallyas and silver-methenamine stainings in paraffin-embedded tissue sections from the same cases used in the immunohistochemical studies.

Brain hemispheres were separated and processed for freezing (for Western blotting and FAAH activity experiments) or for fixation (for immunohistochemistry). Thus, in each case, one of the hemispheres was fixed by immersion in 4% buffered formaldehyde, and select, small tissue blocks containing the areas of interest for this study (hippocampus and entorhinal and parahippocampal cortices) were transferred to 50 mM potassium PBS (KPBS) and cut on a Leica Vibratome. These regions were chosen because they are known to show high densities of β-amyloid peptide (Aβ)-containing neuritic plaques in AD (Morrison and Hof, 2002). Free-floating sections (50 μm thick) were used for immunohistochemical and staining procedures. To obtain a more efficient immunostaining, tissue sections were subjected to an antigen retrieval procedure (Shi et al., 2001). Briefly, sections were placed in a stainless steel pressure cooker containing a boiling solution (0.01 M sodium citrate, pH 6). After they were heated under pressure for 2 min, samples were removed and washed extensively in KPBS.

Western blotting. The protocol used is basically as described previously (Romero et al., 2002). Human brain was obtained at autopsy and a 1 gm piece of cerebral cortical gray matter was homogenized in 10 ml of M-PER mammalian protein extraction reagent (Pierce, Rockford, IL). The homogenate was shaken gently for 10 min and then centrifuged at 27,000 $\times$ *g* for 15 min. The supernatant was isolated, and protein was determined using the BCA protein assay kit (Pierce).

Brain protein extract (50 μg) was reduced and denatured and separated by electrophoresis through a 10.5 $\times$ 10 cm, 0.75-mm-thick 15% polyacrylamide preparative gel. After separation, the proteins in the gel were transferred to nitrocellulose membrane. The nitrocellulose was washed with PBS containing 0.2% Tween 20 (PBST), and remaining binding sites on the membrane were blocked by overnight incubation in PBST containing 2% nonfat dried milk at 4°C. Incubation of primary antibodies was performed at 1:300 dilution in PBST containing 2% nonfat dried milk overnight at 4°C. In some experiments, the antibodies were preincubated with 8 μg/ml of the same immunizing peptides used for the generation of the antibodies. After the nitrocellulose membrane was washed with PBST, it was incubated with an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Sigma, St. Louis, MO), 1:2000 in PBST containing 2% nonfat dried milk for 1 hr at room temperature. The nitrocellulose membrane was washed extensively with PBST, followed by PBS. Finally, the immune complex was visualized by incubating in the presence of nitroblue tetrazolium–5-bromo-4-chloro-3-indoyl phosphate chromogen.

Immunohistochemistry. The protocol used is basically as described previously (Tsou et al., 1998a; Romero et al., 2002), with slight modifications. Briefly, floating sections were washed in KPBS (50 mM), and endogenous peroxidase was blocked by incubation in peroxidase-blocking solution (Dako, Copenhagen, Denmark) for 20 min at room temperature. The sections were then washed in 50 mM KPBS and incubated with the corresponding antibody. The antibodies used included the following: polyclonal anti-CB_1 receptor (1:2000; Affinity Bioreagents), polyclonal anti-CB_2 receptor (1:1500; Affinity Bioreagents, Golden, CO), polyclonal anti-FAAH (Romero et al., 2002) (1:1000), monoclonal anti-CD68 (1:100; Dako) for microglia, and monoclonal anti-GFAP (1:200, Dako) for astrocytes. After incubation with the corresponding primary antibody, the sections were washed in 50 mM KPBS and incubated with biotinylated goat anti-rabbit antibody (1:200), for polyclonal primary antibodies, or biotinylated horse anti-mouse antibody, for monoclonal primary antibodies, at room temperature for 1 hr followed by avidin–biotin complex (Vector Elite, Burlingame, CA), following the manufacturer's instructions. Visible reaction product was produced by treating the sections with 0.04% diaminobenzidine (DAB) (Dako), 2.5% nickel sulfate, and 0.01% H_2O_2 dissolved in 0.1 M sodium acetate.

For double-labeling studies, sections were sequentially incubated with anti-FAAH antibody, treated in the same way as described above, except that the staining was visualized with DAB in the absence of nickel sulfate (rendering brown color) followed by incubation with anti-Aβ monoclonal antibody (1:200; Dako) and signal was revealed with Vector SG (Vector; rendering blue color). The same procedure was used for CB_2/Aβ double-labeling experiments.

Sections were mounted on gelatin-coated slides, dehydrated, and sealed with coverslips. The observations and photography of the slides were done using a Nikon Eclipse E600 microscope and a Nikon FDX-35 camera. Controls for the immunohistochemistry included the preabsorption and coincubation of the antibodies with the corresponding immunogenic proteins (CB_1, fusion protein against amino acids 1–100 of human-CB_1 at 5 μg/ml; CB_2, fusion protein against amino acids 1–33 of human-CB_2 at 5 μg/ml; FAAH, fusion protein against amino acids 561–579 of rat-FAAH at 1.25 μg/ml) and incubation in the absence of primary antibody.

Fatty acid amide hydrolase assay. Frozen tissue sections (50 μm thick) were obtained after cryosectioning. After they were stained with methylene blue, individual neuritic plaques were dissected from entorhinal and parahippocampal cortices under the microscope and transferred to PBS. For control data, portions of similar size were dissected from 50-μm-thick frozen sections of control brain. Afterward, FAAH assays were performed as described (Edgemond et al., 1998). Briefly, plaques were hand homogenized in 50 μl of Tris buffer (50 mM, pH 7.4) containing EDTA (1 mM) and $MgCl_2$ (3 mM). The entire lysate was incubated with [^{14}C]AEA (2000 dpm), labeled on its ethanolamine moiety, for 15 min. The incubation was quenched by the addition of 0.2 ml of chloroform/methanol (1:2) followed by extraction as described (Edgemond et al., 1998). Control incubations were performed in the absence of tissue.

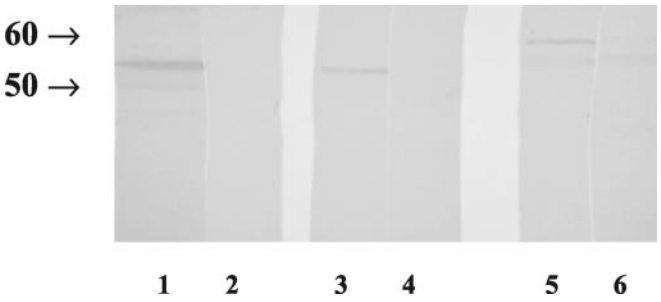

Figure 1. Western blots of CB_1 (lanes 1, 2), FAAH (lanes 3, 4), and CB_2 (lanes 5, 6) immunoreactivities in human temporal cortex from an AD patient. Single bands of ~50 kDa (CB_1 and FAAH) or 60 kDa (CB_2) were observed (lanes 1, 3, and 5, respectively). No immunoreactivities were detected when the primary antibodies were preincubated with the respective immunizing peptides (lanes 2, 4, and 6).

Results

AD diagnosis was confirmed by neuropathological examination on paraffin sections by Gallyas and silver methenamine stainings, revealing the presence of abundant neuritic plaques in entorhinal and parahippocampal cortices (data not shown). Western blots confirmed the presence of CB_1, CB_2, and FAAH in AD homogenates as well as the specificity of the antibodies used (Fig. 1). The bands observed matched the manufacturer's reported weights of the different proteins and previously published data (Tsou et al., 1998b; Romero et al., 2002).

FAAH immunoreactivity in sections from healthy individuals (Fig. 2*A*) revealed the same pattern described previously (Romero et al., 2002), with a predominant labeling of neuronal elements (Fig. 2*A*, inset). Specifically, an intense and regular staining was located on pyramidal neurons in cell bodies and proximal processes. In AD samples, FAAH was detected mainly in cell bodies and processes of hypertrophied astrocytes surrounding neuritic plaques (Fig. 2*B*,*C*) in entorhinal and parahippocampal areas. In contrast, GFAP immunostaining was detectable in both protoplasmic and fibrous astrocytes (Fig. 2*D*). Scattered neurons in the dentate gyrus showed marked FAAH immunoreactivity (data not shown). In support of the immunohistochemical data, FAAH enzyme activity could be detected in five of five individual plaques but never (0 of 5) in tissue pieces of the same size taken from cortices of healthy brain (Fig. 2*F*). Finally, the staining was completely prevented by preabsorption and coincubation with the immunizing peptide (Fig. 2*E*).

No staining for the CB_2 receptor was observed in the same regions of samples from healthy individuals (Fig. 3*A*). In AD samples, CB_2 receptor immunoreactivity was limited to grouped cells, with morphological properties characteristic of neuritic plaque-associated microglia (Fig. 3*B*,*C*). CD68 immunostaining (a commonly used phenotypic marker for all types of microglia) revealed a more abundant signal, including plaque-associated and non-associated microglial cells (Fig. 3*D*). The immunizing peptide reversed the staining for CB_2 (Fig. 3*E*).

Subsequent to these observations and to better characterize the cellular location of FAAH and CB_2 in AD tissue sections, double-immunostaining experiments were conducted. Thus, FAAH immunoreactivity could be clearly seen in hypertrophic astrocytes surrounding Aβ-enriched neuritic plaques (Fig. 4*A*,*B*). In addition, CB_2 immunoreactivity could be circumscribed to Aβ neuritic plaque-associated microglia only (Fig. 4*C*,*D*).

Control brains showed a neuronal pattern of staining for CB_1 receptors, with pyramidal cortical neurons exhibiting a high intensity of labeling (Fig. 5*A*). No changes in the density or location of CB_1 receptors could be seen in the vicinity of neuritic plaques (Fig. 5*B*,*C*). The immunizing peptide was also effective in preventing the immunostaining for CB_1 receptors (Fig. 5*D*).

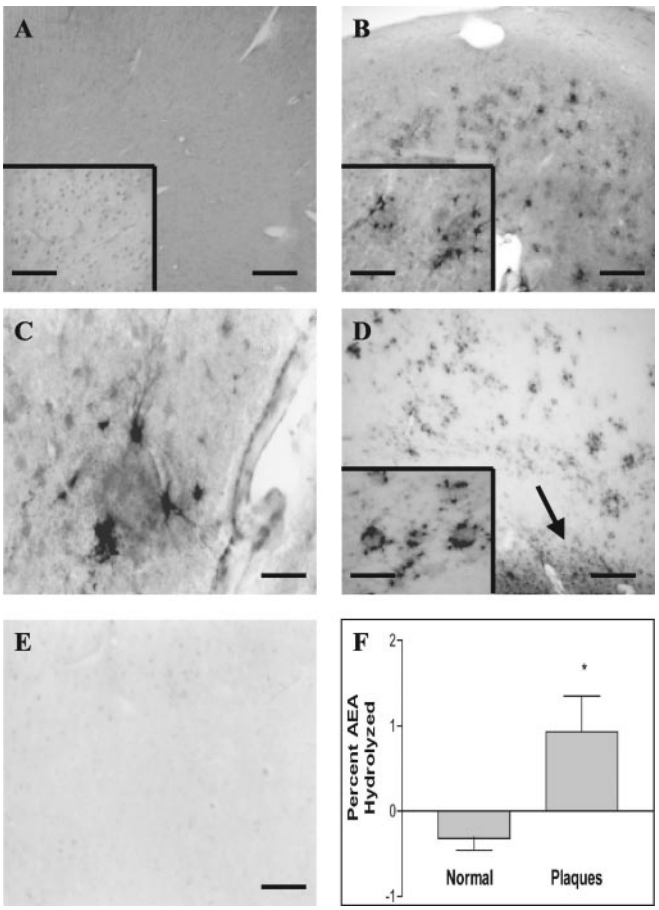

Figure 2. *A–F*, FAAH (*A–C*, *E*) and GFAP (*D*) immunoreactivities in parahippocampal cortex and FAAH activity in neuritic plaques (*F*). *A*, FAAH staining in a healthy individual sample. Note the neuronal pattern of staining (inset). *B*, Low and high (inset) magnifications of FAAH immunoreactivity in parahippocampal cortex of an AD case. Note the intense signal for FAAH in hypertrophied astrocytes surrounding neuritic plaques. *C*, Detail of FAAH immunoreactivity in hypertrophied astrocytes. *D*, Low and high (inset) magnification of GFAP immunoreactivity in an AD case. Note that the signal is detectable in both protoplasmic and fibrous (arrow) astrocytes. *E*, FAAH staining after preabsorption and coincubation of the antibody with the immunizing peptide. Note the absence of any detectable signal. *F*, FAAH activity. Individual plaques were dissected under microscope, homogenized, and assayed for FAAH activity using the conversion of [^{14}C]AEA to [^{14}C]ethanolamine during a 15 min incubation. Shown is the mean of five individual determinations; groups were significantly different using unpaired *t* tests with $p < 0.05$. Scale bars: *A*, *B*, *D*, 800 μm; *E*, inset in *A*, 400 μm; insets in *B* and *D*, 200 μm; *C*, 100 μm.

Discussion

AD is a chronic degenerative disorder of the brain and accounts for the most common form of dementia in the elderly (Strohmeyer and Rogers, 2001). The histopathology of AD is currently well known, with hallmarks including senile plaques, neuritic tangles, loss of neurons, damaged synaptic connections, and reactive gliosis (Giulian, 1999). Reactive gliosis involves both microglia, which attack the senile plaque, and astroglia, which surround the plaque complex and play a critical role in AD inflammation (for review, see Wyss-Coray and Mucke, 2002). Thus, the formation of complex protein aggregates containing Aβ is thought to induce a chronic inflammatory response that

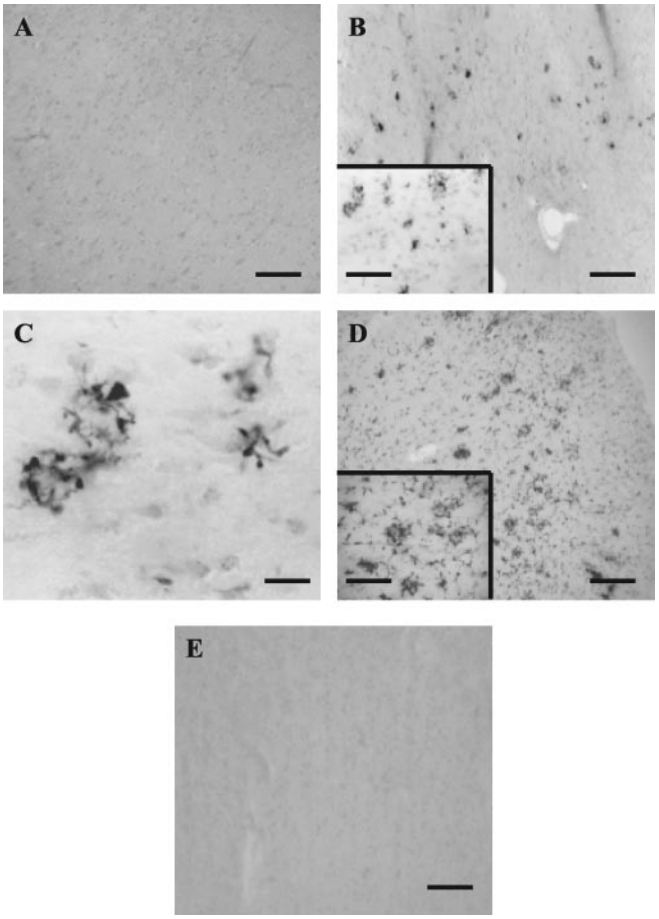

Figure 3. CB_2 (*A–C*, *E*) and CD68 (*D*) stainings in parahippocampal cortex. *A*, CB_2 staining in a healthy individual sample. No detectable signal could be seen. *B*, Low and high (inset) magnifications of CB_2 immunoreactivity in parahippocampal cortex of an AD case. Note the intense signal for CB_2 in microglial cells located on neuritic plaques. *C*, Detail of CB_2 immunoreactivity in neuritic plaque-associated microglia. *D*, Low and high (inset) magnification of CD68 immunoreactivity in an AD case. *E*, CB_2 staining after preabsorption and coincubation of the antibody with the immunizing peptide. Note the absence of any detectable signal. Scale bars: *A*, *B*, *D*, *E*, 800 μm; insets in *B* and *D*, 200 μm; *C*, 100 μm.

leads, among other events, to the activation of both microglia and astroglia. These are known to play a relevant pathophysiological role, because they produce abundant proinflammatory substances that initiate a secondary damaging process (Strohmeyer and Rogers, 2001). The aim of this study was to determine the status of some components of the endocannabinoid system, CB_1 and CB_2 receptors and FAAH, in the brains of AD patients and to explore their possible role in this neurodegenerative disorder.

We report that FAAH protein and activity and CB_2 receptor protein are selectively overexpressed in glial cells that are linked to the inflammatory process that accompanies Alzheimer's disease. To our knowledge, this is the first observation in human tissue that suggests a role of the ECS in the progression of this neurodegenerative disease. We use the term "selectively" in two senses: first, although CB_2 receptors and FAAH exhibit upregulation in glial cells associated with senile plaques, CB_1 receptor density is not modified in the vicinity of these pathological structures. Second, FAAH expression appears to be restricted to reactive astrocytes, and CB_2 receptors are expressed only in activated microglial cells. Whether this upregulation is specific of AD or is common to other pathologies that exhibit reactive gliosis is being investigated currently in our laboratory.

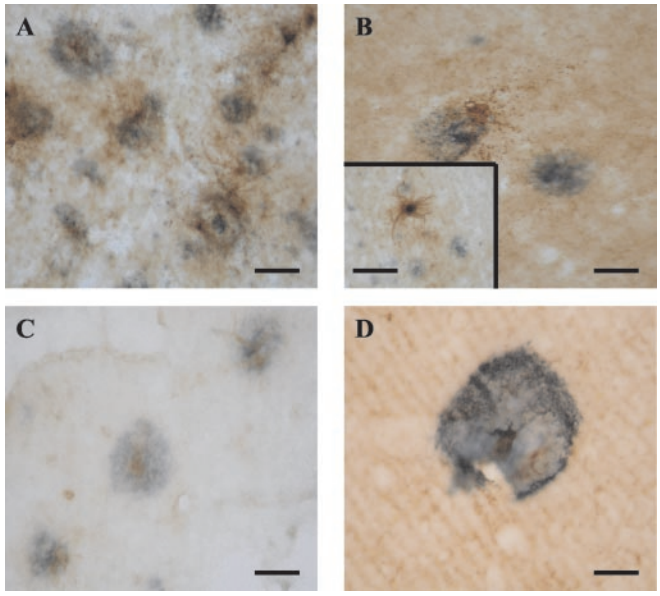

Figure 4. FAAH and CB_2 are expressed in glial cells associated with β-amyloid-enriched neuritic plaques. *A*, *B*, FAAH (brown) and β-amyloid peptide (blue) stainings. Note that FAAH-positive cells are astrocytes surrounding β-amyloid-enriched plaques. *C*, *D*, CB_2 (brown) and β-amyloid peptide (blue) stainings. CB_2 immunostaining is limited to plaque-associated microglial cells.

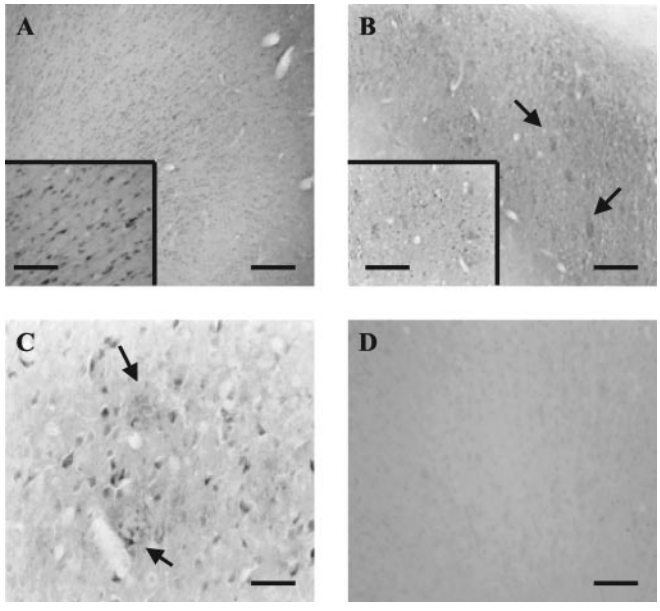

Figure 5. CB_1 staining in parahippocampal cortex. *A*, CB_1 staining in a healthy individual sample. Pyramidal cortical cells showed moderate to intense staining level (inset). *B*, Low and high (inset) magnifications of CB_1 immunoreactivity in parahippocampal cortex of an AD case. Note the general lower intensity of the signal for CB_1 and how the neuritic plaques can be observed easily (arrows). *C*, Detail of CB_1 immunoreactivity, showing no changes in the vicinity of neuritic plaques (arrows). *D*, CB_1 staining after preabsorption and coincubation of the antibody with the immunizing peptide. Note the absence of any detectable signal. Scale bars: *A*, *B*, *D*, 800 μm; inset in *B*, 400 μm; inset in *A*, 200 μm; *C*, 100 μm.

A previous article by Westlake et al. (1994) failed to establish a link between changes in CB_1 receptors or mRNA levels in tissues from Alzheimer's disease patients and the specific pathological events that take place in this illness. In agreement with this report, we have not found changes in the distribution of CB_1 receptors in the vicinity of neuritic plaques; however, it must be noted that the

autoradiographic analysis in the cited study was done using the synthetic cannabinoid CP-55,940 as radioligand. This compound is an agonist for both CB_1 and CB_2 receptor subtypes (Pertwee, 1997), and only decreases in cannabinoid receptor density were detected by these authors. This is in contrast with our observations: although CB_1 receptors were unaltered in the vicinity of the neuritic plaques in our AD samples, CB_2 receptors were dramatically overexpressed in the activated microglia. We do not have a plausible explanation for this discrepancy other than the different methodologies used in each study. It could be argued that the lack of cellular resolution in the autoradiographic method could mask the specific increase in CB_2 receptors that we have observed. Finally, although we did not observe significant changes in CB_1, this subtype of cannabinoid receptors has been implicated in the regulation of microglial function (Waksman et al., 1999), and thus an important role for this subtype of cannabinoid receptors in the inflammatory events related to AD cannot be ruled out.

The presence of FAAH in astrocytes has been observed previously in human CNS (Romero et al., 2002), pointing to a possible role for this enzyme in the regulation of blood vessel tone in the brain and in the regulation of synaptic transmission. It has also been reported that rat astrocytes accumulate and produce anandamide and other acylethanolamides and contain CB_1 receptors (Beltramo and Piomelli, 2000; Walter et al., 2002). Furthermore, we found detectable levels of FAAH activity in the vicinity of individually dissected neuritic plaques, in contrast to control brains. The absence of any activity in control samples may be attributable to the extremely small size of the tissue that was dissected and used in the enzymatic assay.

Because AEA and, at least partially, 2-AG are substrates for FAAH and are both converted to arachidonic acid, the massive presence of FAAH in astrocytes surrounding neuritic plaques suggests that astrocytes, via FAAH, could be a significant source of arachidonic acid and related proinflammatory substances in the vicinity of these plaques, with harmful effects. It is important to note that the use of anti-inflammatory compounds is currently one of the most promising lines of research for the treatment of AD; thus, the beneficial effects of cyclooxygenase inhibitors (such as ibuprofen or aspirin) have recently been reported (In'T Veld et al., 2001; Zandi et al., 2002). In light of these observations, we speculate that inhibition of FAAH activity could be beneficial in preventing the inflammatory process associated with Aβ deposition.

To our knowledge, this report is the first evidence for the presence of CB_2 receptors in the human CNS. It has been reported that CB_2 receptors are expressed in granule and Purkinje cells of the mouse cerebellum (Skaper et al., 1996) and that rat microglial cells express CB_2 receptors and that this expression is upregulated when the microglia become activated (Carlisle et al., 2002). Furthermore, these receptors have recently been reported to play an important role in microglial migration (Walter et al., 2003). It is important to note that we detected CB_2 receptors only in microglial cells, which is in agreement with the well known immunomodulatory effects of CB_2 activation. Thus, many studies have shown that CB_2 receptor activation leads to a myriad of changes in the production of inflammation-related substances, although with results that vary depending on the experimental model used and the concentration of cannabinoids used (Grundy et al., 2001). In any case, the selective presence of CB_2 receptors in microglial cells opens new perspectives on the role of CB_2 receptors in the human CNS and suggests that the modulation of their activity may have therapeutic implications.

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