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# Opposing Control of Cannabinoid Receptor Stimulation on Amyloid-β-Induced Reactive Gliosis: In Vitro and in Vivo Evidence

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## ABSTRACT

Beside cytotoxic mechanisms impacting on neurons, amyloid β (Aβ)-induced astroglial activation is operative in Alzheimer's disease brain, suggesting that persistent inflammatory response may have a role in the illness and that positive results may be achieved by curbing the astroglial reaction. Because the role of the endocannabinoid system could represent a promising field of research, the present study conducted in vitro and in vivo experiments to assess this system. C6 rat astroglioma cells were challenged with 1 μg/ml Aβ 1-42 in the presence or absence of selective agonists and antagonists of cannabinoid (CB)1 and CB2 receptors. Furthermore, rats were inoculated into the frontal cortex with 30 ng of Aβ 1-42 and were i.p. administered with 5 mg/kg of the same substances. Immunohistochemical and biochemical findings revealed that selective agonism at CB1 and antagonism at CB2 receptors was able to blunt Aβ-induced reactive astrogliosis with subsequent overexpression of glial fibrillary acidic protein and S100B protein. Moreover, Aβ provoked down-regulation of CB1 receptors together with a reduction of anandamide concentration, whereas CB2 receptors were up-regulated and 2-arachidonoyl glycerol concentration was increased. Finally, to our knowledge, the current study is the first showing that interactions at cannabinoid receptors result in a dual regulation of Aβ-induced reactive astrogliosis. The data support the assumption that compounds able to selectively block CB2 receptors may have therapeutic potential in controlling Aβ-related pathology, due to their beneficial effects devoid of psychotropic consequences.

Alzheimer's disease (AD), the most common cause of dementia in the elderly, is a significant health problem and its impact is escalating as the human population ages. The characteristic and invariant lesions in brains of afflicted individuals include extracellular accumulation of Aβ fibrils in senile plaques (SP), and intraneuronal fibrillary tangles (Katzman and Saitoh, 1991). These are associated with synaptic dysfunction and neuronal death, especially in limbic and association cortical areas, which have roles in memory and cognitive functioning (Small et al., 2001).

At present, the exact cause accounting for the sporadic cases of AD remains elusive. In the recent past, research has focused on single metabolic disorders or single gene mutations because of the similarities between familial and sporadic AD. However, even if they are phenotypically similar, they seem etiologically different.

Inflammation represents a consistent feature of the AD brain, and mounting evidence suggests that a neuroinflammatory response may be implicated in disease development (Craft et al., 2006). Insoluble deposits of Aβ protein are

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**ABBREVIATIONS:** AD, Alzheimer's disease; SP, senile plaque(s); 2-AG, 2-arachidonoyl glycerol; CB, cannabinoid receptor type; ECS, endocannabinoid system; Aβ, amyloid-β; ACEA, arachidonyl-2′-chloroethylamide; JWH-015, (2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3 carboxamide hydrochloride; SR144528, *N*-((1*S*)-endo-1,3,3-trimethyl bicyclo heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide); RT-PCR, reverse transcription-polymerase chain reaction; OD, optical density; PBS, phosphate-buffered saline; GFAP, glial fibrillary acidic protein; AEA, anandamide; PEA, palmitoylethanolamide; ctrl, control; WIN 55,212, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*)-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone.

strong candidates for initiating the inflammatory response, whereas activated microglia and astrocytes are capable of generating a self-propagating neuroinflammatory process resulting in damage to the brain (Bradl and Hohlfeld, 2003). The inflammatory response may be an attempt to clear Aβ deposits; however, the progressive accumulation of Aβ and its aggregation into insoluble plaques may induce a chronic proinflammatory response leading to compromised neuronal function (Blasko et al., 2004). Indeed, besides cytotoxic mechanisms directly impacting on neurons, Aβ-induced glial activation promotes release of proinflammatory molecules (McGeer and McGeer, 2003; Rosenberg, 2005; Tuppo and Arias, 2005) that may act autocrinally, to self-perpetuate reactive gliosis, and paracrinally, to damage neighboring neurons, thereby amplifying neuropathological lesions (Griffin, 2006). Therefore, determining triggers of the activation and key mediators of this process is crucial to identify suitable targets for effective therapeutic intervention.

In this scenario, interest has been focused on the $Ca^{2+}$/$Zn^{2+}$ astrocyte-derived S100B protein, because compelling evidence considers that it is implicated in the pathogenesis of AD (Marshak et al., 1992; Peskind et al., 2001; Mori et al., 2006). Indeed, S100B has long been associated with numerous neurodegenerative disorders, including AD, where cerebrospinal fluid levels are correlated with cortical atrophy (Petzold et al., 2003). Moreover, S100B overexpression in mice parallel several of the pathological changes occurring in AD, such as dystrophic neurites formation, neuronal toxicity, and learning and memory deficits (Winocur et al., 2001). Studies revealed S100B was overexpressed in activated astrocytes surrounding SP where the degree of glial response correlates with neuritic pathology (Mrak and Griffin, 2001). It has been suggested that its up-regulation may exert damaging effects by promoting the expression of proinflammatory cytokines and inflammatory stress-related enzymes, including the inducible nitric-oxide synthase protein (Hu et al., 1997). Moreover, S100B has been reported to directly increase amyloid precursor protein expression, which, in turn, activates astrocytes and subsequently induces S100B levels to rise (Li et al., 1998), thus fostering a self-sustaining feedback loop that drives the progressive pathological changes observed in AD. This makes a strong case for the importance of this protein in the disease process and suggests that limiting the ongoing reactive gliosis may blunt AD progression.

Several recent studies have suggested that the endocannabinoid system (ECS) participates in brain immune control and neuroprotection, playing a crucial role in the cellular communication network in and between the nervous and immune system during persistent glial activation and neuronal damage. Therefore, the cannabinoid system may represent a new, promising field of research, because many cell types involved in AD neuropathology express components of this system, which can be endogenously or pharmacologically modulated (Pazos et al., 2004). Recent results showing remarkable changes of endocannabinoid levels and receptor concentrations in patients' brains and in experimental models have further reinforced the assumption that this system is substantially dysregulated in AD (Benito et al., 2003). In rat hippocampus, the same authors revealed that Aβ induced a selective 2-fold enhancement of 2-arachidonoyl glycerol (2-AG) along with a significant up-regulation of CB2, but not CB1, receptors concomitant with the onset of neuronal damage markers (van der Stelt et al., 2006). Further evidence, showing that CB1 receptor-selective activation inhibits inducible nitric-oxide synthase expression and blunts tau protein hyperphosphorylation in Aβ-challenged rat neuronal cells (Esposito et al., 2006), has suggested putative neuroprotective and antiinflammatory properties of ECS.

All these findings have encouraged research on the effects of ECS manipulation on Aβ-induced reactive astrogliosis, because it may help identify potential targets that can be used to combat AD progression.

## Materials and Methods

**Rats and Surgical Procedures.** Adult male Wistar rats (300–350 g) were obtained from Harlan (Udine, Italy), and they were housed in a pathogen-free barrier facility under a 12-h light/dark cycle, with ad libitum access to food and water. All experiments were performed in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) and those of the Italian Ministry of Health (D.L. 116/92), and they were approved by the local Institutional Animal Care and Use Committees. Rats ($n = 37$) were anesthetized i.p. with 60 mg/kg pentobarbital. They were then placed in a stereotaxic frame and inoculated with human Aβ peptide fragment (1-42) (Aβ 1-42) (Tocris Cookson, Inc., Bristol, UK) into the frontal cortex (coordinates relative to the bregma: AP, +3.2 mm; ML, −2 mm; DV, −3 mm). The peptide was dissolved in artificial cerebrospinal fluid to the concentration of 10 μg/ml, and 3 μl was injected using a microdialysis pump, keeping the flow to the constant value of 0.5 μl/min. Control rats ($n = 8$) were treated according to the same procedure, and they were inoculated with an equivalent volume of artificial cerebrospinal fluid.

An aliquot of Aβ 1-42 solution was subjected to SDS-polyacrylamide gel electrophoresis, and the peptide was detected in an intermediate form between the monomeric and dimeric forms, but not in the fibrillary form (data not shown).

**In Vivo Treatment.** On days 3, 5, and 7 postsurgery, rats were given one of the following CB receptor ligands at 5 mg/kg i.p.: CB1-selective agonist arachidonyl-2′-chloroethylamide ACEA (CB1 $K_i$ value = 1.4 nM; CB2 $K_i$ value >2000 nM; Hillard et al., 1999) and CB2-selective agonist JWH-015 (CB1 $K_i$ value = 383 nM; CB2 $K_i$ value = 13.8 nM; Showalter et al., 1996), both from Tocris Cookson, Inc.; CB1-selective antagonist SR141716A (CB1 $K_i$ value = 1.98 nM; CB2 $K_i$ value >1000 nM; Rinaldi-Carmona et al., 1994) and CB2-selective antagonist SR144528 (CB1 $K_i$ value = 437 nM; CB2 $K_i$ value = 0.6 nM; Rinaldi-Carmona et al., 1998), both kindly provided by Dr. Madaleine Mosse (Sanofi-Recherche, Montpellier, France). Control rats were i.p. given an equivalent volume of the proper vehicle.

On the 7th day, rats to be subjected to immunoblotting and RT-PCR experiments were sacrificed by decapitation, and the extracted brains frozen in liquid $N_2$. Rats to be subjected to morphological analysis were sacrificed through transcardiac perfusion with 4% paraformaldehyde/0.1% glutaraldehyde. The extracted brains were postfixed overnight with the same fixative, protected with 30% sucrose, and frozen using 2-methylbutane.

**C6 Rat Glioma Cell Cultures.** C6 rat glioma cells (ATCC CCL-107) were cultured in 10 cm i.d. Petri dishes in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% $CO_2$, 95% air.

Confluent cells were treated with 1 μg/ml Aβ 1-42 in the presence or absence of the following substances: the selective CB1 agonist ACEA ($10^{-6}$–$10^{-8}$ M), the selective CB2 agonist JWH-015 ($10^{-6}$–$10^{-8}$ M), the selective CB1 antagonist SR141716A ($10^{-7}$ M), and the selective CB2 antagonist SR144528 ($10^{-7}$ M). After a variable incu-

bation time, cells were subjected to the different treatments, as described below.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-Formazan Assay.** Cells were plated at the density of $1 \times 10^5$ cells/well, left to adhere for 2 h at 37°C, and then treated as described previously. After 48-h incubation time, cells were added with 25 $\mu$l of 5 g/l 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium solution. Cells were incubated for an additional 3 h at 37°C before being lysed, and then dark blue crystals were solubilized with 125 $\mu$l of a 50% *N*,*N*-dimethyl formamide/20% sodium dodecyl sulfate solution with an adjusted pH of 4.5. Optical density (OD) was measured with a spectrophotometer (Titertek Multiskan MCC/340; Titertek Instruments, Huntsville, AL) equipped with a 620-nm filter. Cell viability was calculated as OD treated/OD control ratio.

**Western Blot Analysis.** Western blot analysis was performed on rat hippocampus homogenates and on extracts of C6 cells, both treated as described previously.

Twenty-four hours after treatment, harvested cells ($1 \times 10^6$ cells) were washed twice with ice-cold PBS, and then it was centrifuged at 180*g* for 10 min at 4°C. The pellet was resuspended in 50 $\mu$l of ice-cold hypotonic lysis buffer and incubated on ice for additional 15 min. Ipsi- and contralateral hippocampi were dissected from frozen brains and lysed in the same manner.

Cells or hippocampi were then mechanically lysed, and the total protein extract was obtained by centrifugation at 13,000*g* for 15 min at 4°C. Samples were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were transferred onto nitrocellulose membrane and incubated with one of the following antibodies: rabbit anti-GFAP (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse anti-S100B (1:250; Abcam, Cambridge, UK), rabbit anti-CB1 (1:250, ABR-Affinity BioReagents, Golden, CO), rabbit anti-CB2 (1:250; Cayman Chemical, Ann Arbor, MI), and mouse anti-$\beta$ actin (1:1000; Lab Vision, Fremont, CA). Appropriate peroxidase-conjugated secondary antibodies (1:1000; Dako Denmark A/S, Glostrup, Denmark) were used, and proteins were visualized using an enhanced chemiluminescence kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Protein expression was quantified by densitometric scanning of the X-ray films with a GS 700 imaging densitometer (Bio-Rad, Hercules, CA) and a computer program (Molecular Analyst; IBM, White Plains, NY).

**mRNA Analysis.** mRNA level was determined using the semiquantitative RT-PCR method in C6 cells and in rat hippocampi, both treated as described previously. C6 cells were analyzed 12 h following A$\beta$ 1-42 challenge.

Total mRNA was extracted by TRIzol reagent (Invitrogen, Milan, Italy), and its concentration and purity were determined from the A260/A280 ratio using a UV spectrophotometer DU40 (Beckman Coulter, Fullerton, CA).

The primers sequences used for amplification were as follows: GFAP sense, 5′-GAAGCAGGGCAAGATGGAGC-3′, and antisense, 5′-AGGTTGGTTTCATCTTGGAG-3′, S100B sense, 5′-GGTGACAAGCACAAGCTGAA-3′, and antisense, 5′-TGGACGAAGGCCATAAAC-3′; CB1 receptor sense, 5′-GATGTCTTTGGGAAGATGAACAAGC-3′, and antisense, 5′-AGACGTGTCTGTGGACACAGACATGG-3′; CB2 receptor sense, 5′-TA(C/T)CC(G/A)CCT(A/T)CCTACAAAGCTC-3′, and antisense, 5′-ATGAAGATCCTGACCGCGCGT-3′; and $\beta$-actin sense, 5′-ATGAAGATCCTGACCGCGCGT-3′, and antisense, 5′-AACGCAGCTCAGTAACAGTCCG-3′.

One microgram of total mRNA from each sample was analyzed using a SuperScript One Step RT-PCR with Platinum *Taq* kit (Invitrogen, San Giuliano Milanese, Italy) in a total reaction volume of 25 $\mu$l. Polymerase chain reaction products were electrophoresed on 1% agarose gel, and they were visualized with ethidium bromide. The integrated density values of the bands representing amplified products were acquired and analyzed by GS 700 imaging densitometer (Bio-Rad) and a computer program (Molecular Analyst; IBM).

**Measurement of Endocannabinoid Levels.** C6 cells challenged with 1 $\mu$g/ml A$\beta$ 1-42 were added with ice-cold methanol and 100 pmol of $d^8$-anandamide (AEA), $d^4$-palmitoylethanolamide (PEA), and $d^5$-2-AG as internal standards. Cells were then extracted three times with chloroform by 30-s sonication. The three organic phases were then pooled, lyophilized, and prepurified by open bed chromatography on silica gel, eluted with chloroform/methanol (9:1). The eluate was then lyophilized and analyzed by isotope dilution-liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. Mass spectrometric detection was carried out in the selected ion monitoring mode using *m*/*z* values of 356.0 and 348.0 (molecular ions + 1 for deuterated and undeuterated AEA), 384.35 and 379.35 (molecular ions + 1 for deuterated and undeuterated 2-AG), and 304.0 and 300.0 (molecular ions + 1 for deuterated and undeuterated PEA). The area ratios between signals of deuterated and undeuterated AEA varied linearly with varying amounts of undeuterated AEA (30 fmol–100 pmol). The same applied to the area ratios between signals of deuterated and undeuterated 2-AG in the 100 pmol-to-20 nmol interval. AEA, 2-AG, and PEA levels in unknown samples were calculated on the basis of their area ratios with the internal deuterated standard signals area. The amount of endocannabinoids is expressed as picomoles per milligram of lipid extract.

**Immunohistochemistry.** Brains were cut on a freezing sliding microtome (Leica SM 2000 R; Leica, Milan, Italy) to obtain 30-$\mu$m sections collected in a 15 mM $NaN_3$ PBS solution. Sections were stored at 4°C.

Sections mounted upon gelatin-coated slides were treated with Nissl staining solution to reveal tissue basic morphology and with Fluoro Jade-B (Histo-Chem, Jefferson, AR), an anionic fluorescein useful for the staining of neurons undergoing degeneration (Schmued and Hopkins, 2000).

Immunohistochemistry reactions were performed on hippocampal and cortical sections adjacent to the site of the injection, obtained from both control and treated rats. Free-floating sections were permeabilized and blocked with PBS containing 15 mM $NaN_3$, 10% albumin from bovine serum, and 0.25% Triton X-100, and then they were incubated with one of the following primary antibodies at 4°C in a damp chamber by continuous shaking: mouse anti-GFAP (1:400, Sigma-Aldrich, St. Louis, MO), mouse anti-S100B (1:1000; Sigma-Aldrich), and mouse anti-CD 68 (clone ED1; Serotec, Oxford, UK). After two overnight incubation, the sections were treated with the proper biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA) and with the preformed avidin-biotinylated peroxidase complex (VECTASTAIN ABC kit; Vector Laboratories), and the reaction was revealed by 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Sections were mounted upon gelatin-coated slides before coverslipping with a nonaqueous medium.

Slides were analyzed with a Zeiss Axioskope 2 light microscope (Carl Zeiss, Oberkochen, Germany), and images were captured at 4× magnification by means of a high-resolution digital camera (C4742-95; Hamamatsu Photonics, Milan, Italy) and HiPic software (Hamamatsu Photonics). Quantification analysis was performed with a MCID software (Imaging Research, St. Catherine's, ON, Canada).

**Statistical Analysis.** Results were expressed as mean ± S.E.M. of experiments. Statistical analysis was performed using parametric one-way analysis of variance, and multiple comparisons were performed by Bonferonni's test. Values of $p < 0.05$ were considered significant.

## Results

**Effect of CB Receptor Agonism and Antagonism on A$\beta$-Induced C6 Cell Proliferation.** The first set of experiments was devoted to investigate whether interactions at CB1 and CB2 receptors could affect C6 cell proliferation induced by A$\beta$. Hence, C6 cells were exposed to 1 $\mu$g/ml A$\beta$ 1-42 and 48 h later it was observed that cell proliferation was

markedly enhanced in the treated cells compared with the unexposed cells. Such increase was significantly reverted, in a concentration-dependent manner, if C6 cells were challenged with ACEA ($10^{-8}$–$10^{-6}$ M), this being a potent and selective agonist at the CB1 receptor (Hillard et al., 1999). Similarly, the CB2 receptor-selective antagonist SR144528, at the concentration of $10^{-7}$ M, was able to blunt C6 cell proliferation promoted by Aβ. Conversely, under the same experimental conditions, Aβ-induced C6 cell proliferation was greatly amplified by exposure to $10^{-7}$ M CB1 receptor-selective antagonist SR141716A, and to the CB2 receptor-selective agonist JWH-015, depending on the concentration ranging from $10^{-8}$ to $10^{-6}$ M (Fig. 1). Taken together, these data are consistent with the assumption that stimulation exerted at CB1 or antagonism at CB2 receptors results in a clear-cut attenuation of C6 cell proliferation provoked by exposure to Aβ.

**Effect of CB Receptor Agonism and Antagonism on Aβ-Induced GFAP and S100B Protein Transcription and Expression.** Another set of experiments was aimed at assessing the potential modulating role of cannabinoid signals on GFAP and S100B protein transcription and expression induced by Aβ. First, the selective agonists and antagonists of cannabinoid receptors were tested, both in unchallenged C6 cells and the hippocampus of untreated rats, to assess whether they could affect astroglial function in the absence of Aβ 1-42. These experiments showed that none of the four compounds influenced GFAP and S100B protein expression (data not shown).

Subsequently, the effects of the same substances on these parameters were investigated both in extracts of C6 cells, challenged with 1 μg/ml Aβ 1-42, and in hippocampus homogenates of rats inoculated with 30 ng of Aβ 1-42.

In vitro experiments showed that concentrations of either $10^{-7}$ M of the selective CB1 receptor agonist ACEA, or the selective CB2 receptor antagonist SR144528, were able to significantly reduce GFAP and S100B protein transcription and expression at 12 and 24 h following Aβ challenge, respectively. On the contrary, an opposing effect was reported when the CB2 receptor was stimulated with $10^{-7}$ M of the selective agonist JWH-015, and when the CB1 receptor was antagonized with $10^{-7}$ M of the selective antagonist SR141716A (Figs. 2 and 3).

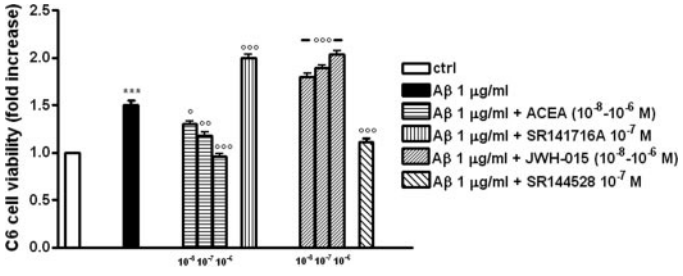


**Fig. 1.** Effect of Aβ on C6 rat glioma cell proliferation. Cells were challenged with 1 μg/ml Aβ 1-42 in the presence or absence of one of the following substances: CB1-selective agonist ACEA ($10^{-8}$–$10^{-6}$ M), CB1-selective antagonist SR141716A ($10^{-7}$ M), CB2-selective agonist JWH-015 ($10^{-8}$–$10^{-6}$ M), and CB2-selective antagonist SR144528 ($10^{-7}$ M). Forty-eight hours later cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-formazan assay. Results are expressed as cell viability -fold increase versus unchallenged (ctrl) or Aβ-challenged cells (Aβ). Results are the mean ± S.E.M. of four experiments in triplicate. ***, $p < 0.001$ versus ctrl; °, $p < 0.05$; °°, $p < 0.01$; and °°°, $p < 0.001$ versus Aβ.

In vivo experiments detected a significant increase of both GFAP and S100B protein transcription and expression in the hippocampus of Aβ-injected rats, whereas no effect was observed in the vehicle inoculated control rats 7 days following surgical procedures. Intraperitoneal treatment with 5 mg/kg of the selective CB1 receptor agonist ACEA, or the selective CB2 receptor antagonist SR144528, generated a significant reduction of Aβ-induced hippocampal GFAP and S100B protein transcription and expression. On the contrary, the same dose of i.p.-administered JWH-015 (selective CB2 agonist), or SR141716A (selective CB1 antagonist), provoked a clear increase in the hippocampal transcription and expression of GFAP and S100B protein, compared with rats inoculated with Aβ but i.p. injected with the vehicle (Figs. 2 and 3). This set of findings suggests a dual modulating role operated by CB receptor signals on Aβ-induced hippocampal reactive astrogliosis, as expressed by changes occurring in GFAP and S100B transcription and expression.

**Effect Induced by Aβ Challenge on CB1 and CB2 Receptor Transcription and Expression and Also on Endocannabinoid Concentration Levels.** Further experiments were aimed at investigating whether exposure to Aβ was able to affect cannabinoid receptor transcription and expression, and to provoke changes in endocannabinoid concentration levels. Transcription and expression of CB receptors were evaluated both in extracts of C6 cells stimulated with 1 μg/ml Aβ 1-42, and in hippocampus homogenates of rats inoculated with 30 ng of Aβ 1-42. Compared with the respective controls, both in vitro and in vivo Aβ treatment significantly reduced CB1 and clearly up-regulated CB2 receptor transcription and expression 12 and 24 h after treatment, respectively (Table 1).

Endocannabinoid levels were measured in extracts of C6 cells 24 h following 1 μg/ml Aβ 1-42 challenge. The findings showed a decrease in preferential CB1 agonist AEA levels, together with a parallel increase in CB1/CB2 agonist 2-AG concentrations, in comparison with unexposed cells. On the contrary, "endocannabinoid-like" PEA concentrations were unaffected by the presence of Aβ (Table 2).

Taken together, these results confirm the occurrence of marked and selective Aβ exposure-induced ECS alterations, suggesting that this system is definitely involved in the pathophysiology of Aβ-related neurodegenerative processes.

**Immunohistochemistry.** Microscopic examination of sections obtained from controls and treated rats showed neither histological alterations nor cellular damages, whereas it revealed significant reactive astrogliosis expressed by the increased GFAP and S100B expression observed in the ipsilateral hippocampal sections of treated rats compared with controls. However, immunohistochemical procedures using anti-CD68 antibody, failed to demonstrate any microglial response in the hippocampus far from the frontal cortex site of Aβ injection (data not shown).

Furthermore, both the selective CB1 receptor agonist ACEA and the selective CB2 receptor antagonist SR144528 blunted Aβ-induced GFAP and S100B protein up-regulation, as shown in Figs. 4 and 5. SR144528 resulted more effective, supporting the hypothesis that changes in CB2 receptor functioning may be primarily involved in the astrogliosis process occurring in Aβ toxicity.

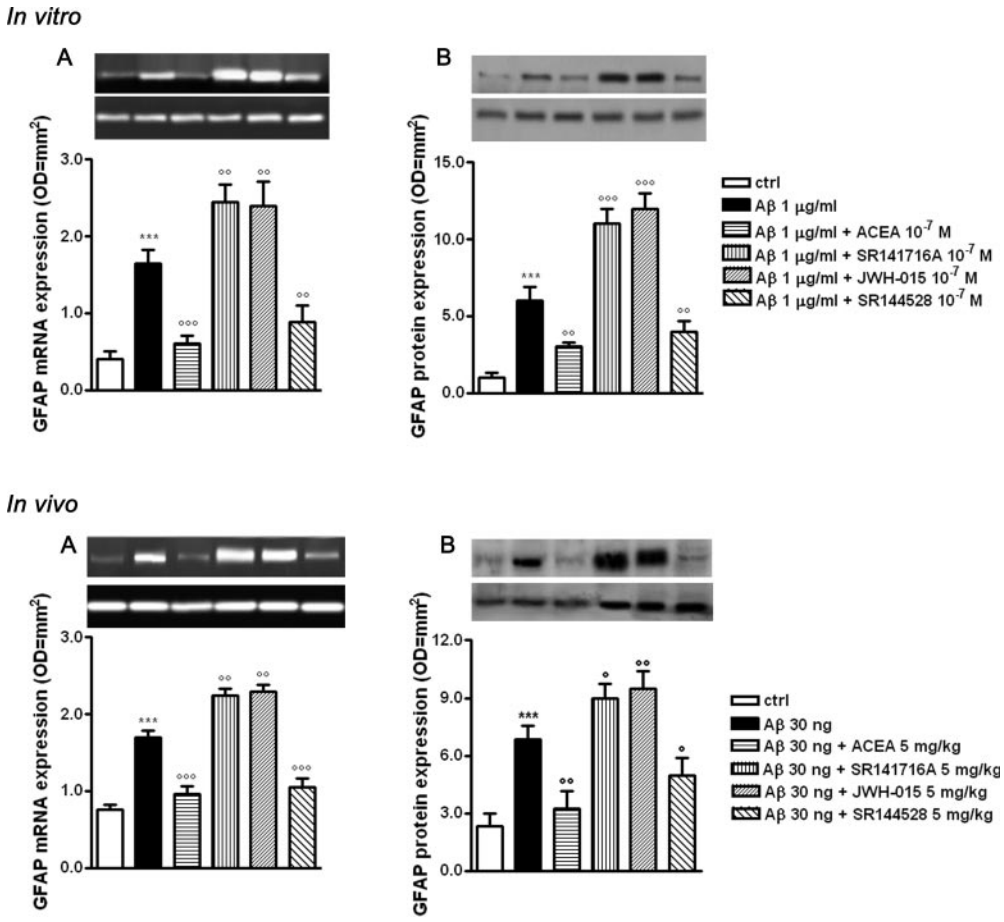


**Fig. 2.** Effect of CB receptor agonism and antagonism on Aβ-induced GFAP protein transcription and expression in vitro and in vivo. In vitro experiments. C6 rat glioma cells were challenged with 1 μg/ml Aβ 1-42 in the presence or absence of one of the following substances ($10^{-7}$ M): CB1-selective agonist ACEA, CB1-selective antagonist SR141716A, CB2-selective agonist JWH-015, and CB2-selective antagonist SR144528. GFAP mRNA and protein expression was evaluated 12 and 24 h following Aβ challenge, respectively. A, results of GFAP RT-PCR amplification and densitometric analysis of corresponding bands. B, results of GFAP protein Western blot analysis and densitometric analysis of corresponding bands. Results are the mean ± S.E.M. of three separate experiments. ***, $p < 0.001$ versus unchallenged cells (ctrl); °°, $p < 0.01$; and °°°, $p < 0.001$ versus Aβ-challenged cells. In vivo experiments. Rats were inoculated into the frontal cortex with 30 ng of Aβ 1-42 and i.p.-treated with one of the following substances (5 mg/kg): CB1-selective agonist ACEA, CB1-selective antagonist SR141716A, CB2-selective agonist JWH-015, and CB2-selective antagonist SR144528. Seven days after inoculation, hippocampal level of GFAP mRNA and protein expression were evaluated. A, results of GFAP RT-PCR amplification and densitometric analysis of corresponding bands. B, results of GFAP protein Western blot analysis and densitometric analysis of corresponding bands. Results are the mean ± S.E.M. of three separate experiments. ***, $p < 0.001$ versus vehicle inoculated rats (ctrl); °, $p < 0.05$; °°, $p < 0.01$; and °°°, $p < 0.001$ versus Aβ-inoculated rats.

## Discussion

The current report demonstrates that Aβ exposure induces substantial changes in ECS, both in vitro and in vivo. In C6 cell culture, CB1 receptor transcription and expression decreased significantly, and they were flanked by a clear reduction in the concentration of its preferential endogenous ligand AEA, whereas CB2 receptor mRNA and protein levels and the concentration of its ligand 2AG (which can also activate CB1 receptor) were up-regulated. Such results were replicated in vivo with regard to CB receptor transcription and expression.

The findings reported herein agree with our previous data (van der Stelt et al., 2006) and with those showing that CB2 receptor expression markedly increases following insults that provoke neuroinflammatory processes, such as those occurring in several chronic neurodegenerative disorders (Fernández-Ruiz et al., 2005). At present, although the pathophysiological significance of these changes remains unclear, it is noteworthy that they are associated with alterations of cytokine production and astroglial activation. The enhancement of 2-AG concentrations is probably due to its increased biosynthesis, since our previous study (van der Stelt et al., 2006) revealed that levels of the mRNA encoding the 2-AG synthesizing enzyme diacylglycerol lipase were also augmented following Aβ exposure. These findings agree with others showing raised diacylglycerol lipase enzymatic activity in the hippocampus of AD patients (Farooqui et al., 1988), and the latter suggests that a tissue-selective up-regulation of 2-AG also occurs during this disorders in humans.

All these data showing significant Aβ-induced ECS involvement in the inflammatory processes suggest that manipulation of the endocannabinoid signaling apparatus may modulate astrogliosis and release of proinflammatory molecules observed in response to Aβ exposure. Furthermore, the observation that astrocytes are able to produce endocannabinoids, display cannabinoid receptors (Walter and Stella, 2004), and also possess the molecular machinery to inactivate these substances (Beltramo et al., 1997) provided evi-

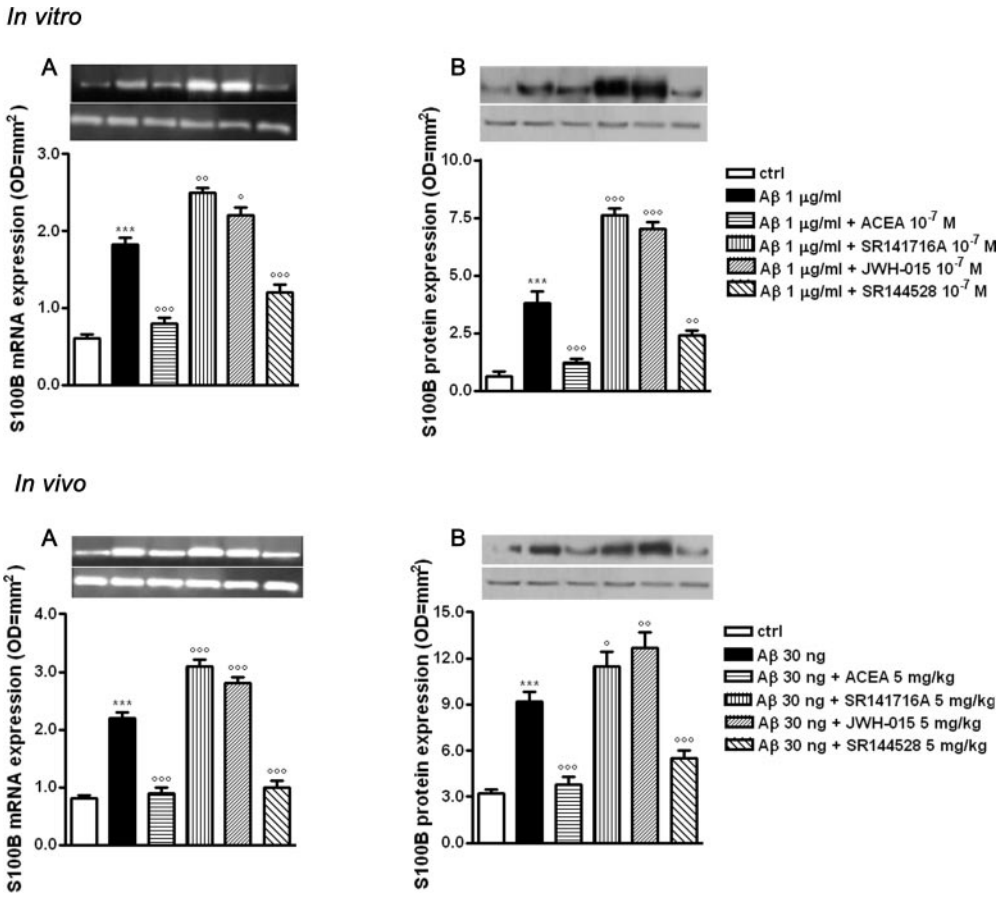


**Fig. 3.** Effect of CB receptor agonism and antagonism on Aβ-induced S100B protein transcription and expression in vitro and in vivo. In in vitro experiments, C6 rat glioma cells were challenged with 1 μg/ml Aβ 1-42 in the presence or absence of one of the following substances ($10^{-7}$ M): CB1-selective agonist ACEA, CB1-selective antagonist SR141716A, CB2-selective agonist JWH-015, and CB2-selective antagonist SR144528. S100B mRNA and protein expression was evaluated 12 and 24 h after Aβ challenge, respectively. A, results of S100B RT-PCR amplification and densitometric analysis of corresponding bands. B, results of S100B protein Western blot analysis and densitometric analysis of corresponding bands. Results are the mean ± S.E.M. of three separate experiments. ***, $p < 0.001$ versus unchallenged cells (ctrl); °, $p < 0.05$; °°, $p < 0.01$; and °°°, $p < 0.001$ versus Aβ-challenged cells. In in vivo experiments, rats were inoculated into the frontal cortex with 30 ng of Aβ 1-42, and i.p.-treated with one of the following substances (5 mg/kg): CB1-selective agonist ACEA, CB1-selective antagonist SR141716A, CB2-selective agonist JWH-015, and CB2-selective antagonist SR144528. Seven days after inoculation, hippocampal level of S100B mRNA and protein expression were evaluated. A, results of S100B RT-PCR amplification and densitometric analysis of corresponding bands. B, results of S100B protein Western blot analysis and densitometric analysis of corresponding bands. Results are the mean ± S.E.M. of three separate experiments. ***, $p < 0.001$ versus vehicle-inoculated rats (ctrl); °, $p < 0.05$; °°, $p < 0.01$; and °°°, $p < 0.001$ versus Aβ-inoculated rats.

TABLE 1

Effect of Aβ on CB1 and CB2 receptor transcription and expression in vitro and in vivo

For in vitro experiments, cells were challenged with 1 μg/ml of Aβ 1-42. Twelve hours later, quantification of CB1 and CB2 receptor mRNA was carried out by RT-PCR, whereas quantification of the same protein expression was performed by Western blot analysis 24 h following Aβ challenge. For in vivo experiments, rats were inoculated into the frontal cortex with 30 ng of Aβ 1-42. Seven days later, quantification of hippocampal CB1 and CB2 receptor mRNA was carried out by RT-PCR, whereas quantification of the same hippocampal protein expression was performed by Western blot analysis. Data are expressed as optical density, and they are the mean ± S.E.M. of three separate experiments.

| | CB1 mRNA Expression | CB1 Protein Expression | CB2 mRNA Expression | CB2 Protein Expression |
|---|---|---|---|---|
| In vitro experiments | | | | |
| Unchallenged cells | 0.59 ± 0.05 | 1.20 ± 0.029 | 0.34 ± 0.02 | 0.50 ± 0.09 |
| Aβ (1 μg/ml)-challenged cells | 0.3 ± 0.06*** | 0.5 ± 0.04*** | 0.52 ± 0.03*** | 1.24 ± 0.12*** |
| In vivo experiments | | | | |
| Vehicle-inoculated rats | 1.95 ± 0.08 | 0.62 ± 0.02 | 1.19 ± 0.19 | 0.46 ± 0.02 |
| Aβ (30 ng)-inoculated rats | 0.92 ± 0.046°°° | 0.35 ± 0.026°°° | 2.59 ± 0.157°°° | 0.85 ± 0.026°°° |

*** $p < 0.001$ vs. unchallenged cells.
°°° $p < 0.001$ vs. vehicle-inoculated rats.

dence for an astroglial endocannabinoid signaling system (Pazos et al., 2005), which could be a possible target for modulating reactive astrogliosis and subsequent inflammatory response. This assumption is supported by recent reports that cannabinomimetics acting on astroglial cells induced antiinflammatory effects that involved nitric oxide signaling (Esposito et al., 2001). Moreover, studies showed that administration of WIN 55,212-2, a synthetic CB1/CB2 receptor agonist, was able to blunt the release of inflammatory mediators by human astrocytes (Sheng et al., 2005), and these results further reinforced the notion that cannabinoids can modulate astroglial activation.

TABLE 2

Effect of Aβ on endocannabinoid levels in C6 rat glioma cells

Measurement of endocannabinoid levels in C6 rat glioma cells 24 h after 1 μg/ml Aβ 1-42 challenge. The amount of endocannabinoids is expressed as picomoles per milligram of lipid extract. Results are the mean ± S.E.M. of four separate experiments in triplicate.

| Treatment | Lipid | | |
|---|---|---|---|
| | AEA | 2-AG | PEA |
| | *pmol/mg* | | |
| ctrl | 3.2 ± 0.05 | 105 ± 11 | 32 ± 1.6 |
| Aβ 1 (μg/ml) | 0.8 ± 0.02* | 153 ± 16* | 33 ± 2 |

* $p < 0.05$ vs. unchallenged cells (ctrl).

To our knowledge, the present study is the first to show that interactions at cannabinoid receptors may regulate, in a dual manner, aspects of brain response to Aβ exposure, such as the release of the potentially toxic S100B protein and astroglial response modulation. Indeed, although astrocytes generally play a neuroprotective role in the physiology of the central nervous system, Aβ-activated astrocytes in the AD brain are assumed to be implicated in a chronic, self-sustained inflammatory response through the release of mediators responsible for the cellular damage (Griffin et al., 1998).

The current findings demonstrated that agonism at CB1 receptor weakened astrocytic activation and subsequent GFAP and S100B protein up-regulation in vitro, whereas agonism exerted at CB2 intensified these effects, suggesting that ECS plays an opposing role in the control of astroglial response to Aβ.

These results were further validated by the evidence achieved through the selective blockade of cannabinoid receptors, in that antagonism at CB1 receptor amplified, whereas that operated at CB2 blunted the reactive astrogliosis with the associated overexpression of both GFAP and S100B protein. Moreover, the data obtained in vivo from hippocampus homogenates of Aβ-inoculated rats fully paralleled the above-described results obtained in vitro.

The effects provoked by receptor blockade interestingly point to the existence of a tonic control operated by ECS upon astroglial functioning, at least in the conditions of Aβ-induced reactive astrogliosis, as further confirmed by immunohistochemical studies carried out into hippocampus ipsilateral to the site of Aβ injection. The morphological findings confirmed the occurrence of a reactive astrogliosis, with related increase in GFAP and S100B protein expression far from the frontal cortex where the peptide was administered. This may be because astrocytes, but not microglia, work as a syncytium of interconnected cells, both in health and in disease, and this is why immunohistological procedures failed to reveal any distal involvement of microglial cells. These immunohistochemical studies confirmed that hippocampal astrogliosis induced by Aβ cortical injection, as well as the related increase in GFAP and S100B expression, was significantly reduced by i.p. treatments with both the CB1-selective agonist ACEA and the CB2-selective antagonist SR144528.

The results showing that S100B levels were increased in the hippocampus of Aβ-injected rats and that they were restored following interactions at cannabinoid receptors are especially promising in view of the role of this protein as key modulator of astrogliosis. Prolonged elevation of S100B produces concern because it may determine Aβ-induced detrimental effects via its proinflammatory activity, in addition to its facilitating role in inappropriate neuritogenesis and amyloid precursor protein overexpression (Li et al., 1998). Therefore, if S100B acts as an important perpetrator in the exacerbation of Aβ neurotoxicity, any treatment able to restore homeostatic levels of S100B could give rise to beneficial consequences, and it may represent a novel therapeutic tool able

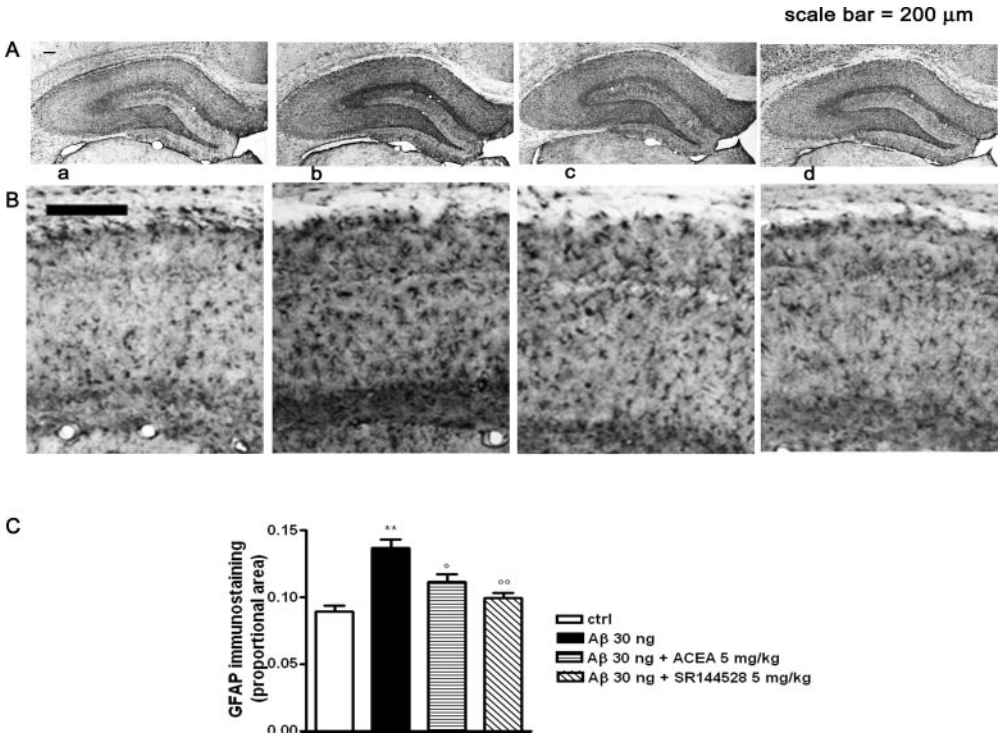


**Fig. 4.** Photomicrographs of rat hippocampus showing the effect of CB1 agonism and CB2 antagonism on Aβ-induced GFAP expression. Rats were inoculated with 30 ng of Aβ 1-42 and i.p.-treated with 5 mg/kg of the selective CB1 agonist ACEA or the selective CB2 antagonist SR144528. Seven days later, immunohistochemical reactions were performed. Representative photomicrographs of the whole ipsilateral rat hippocampus (A) and the corresponding CA1 region labeled with anti-GFAP antibody (B), showing astroglial cell immunostaining. a, vehicle-inoculated rats (ctrl). b, Aβ-inoculated rats. c, rats inoculated with Aβ and i.p.-treated with ACEA. d, rats inoculated with Aβ and i.p.-treated with SR144528. Magnification, 4×. C, quantification of astroglial cell density as the proportional area immunostained with anti-GFAP antibody, i.e., the area occupied by the positively staining cells compared with the total scanned area. Data are shown as mean ± S.E.M. of five experiments. **, $p < 0.01$ versus ctrl; °, $p < 0.05$; and °°, $p < 0.01$ versus Aβ-inoculated rats.

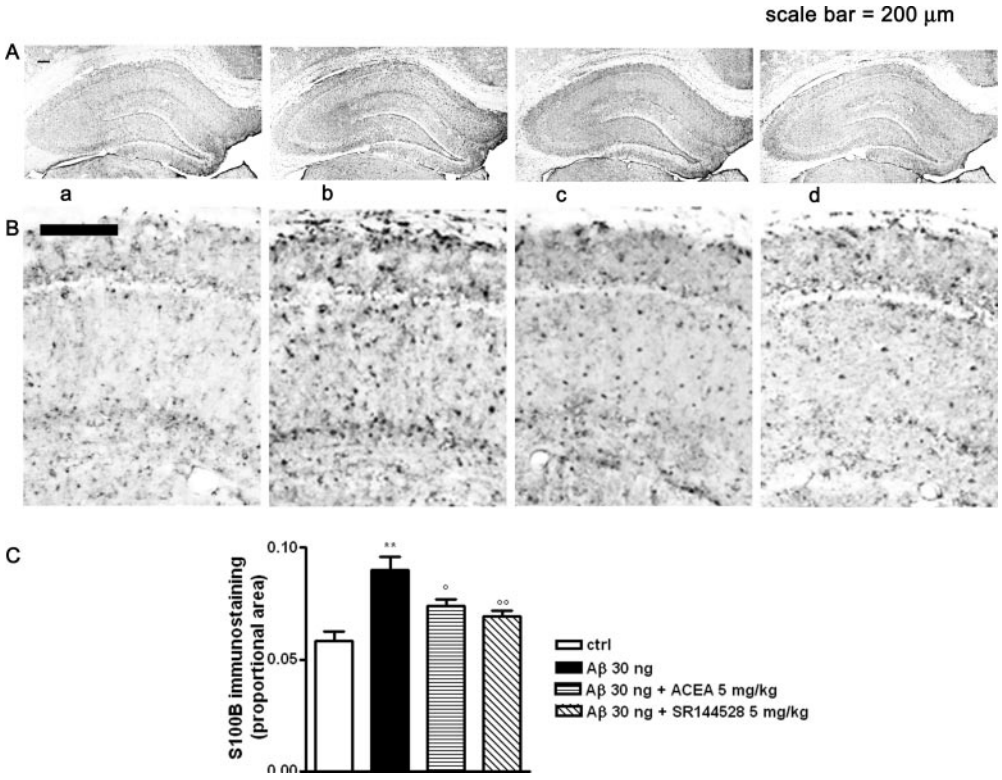


**Fig. 5.** Photomicrographs of rat hippocampus showing the effect of CB1 agonism and CB2 antagonism on Aβ-induced S100B expression. Rats were inoculated with 30 ng of Aβ 1-42 and i.p.-treated with 5 mg/kg of the selective CB1 agonist ACEA or the selective CB2 antagonist SR144528. Seven days later, immunohistochemical reactions were performed. Representative photomicrographs of the whole ipsilateral rat hippocampus (A) and the corresponding CA1 region (B) labeled with anti-S100B antibody, showing astroglial cell immunostaining. a, vehicle-inoculated rats (ctrl). b, Aβ-inoculated rats. c, rats inoculated with Aβ and i.p.-treated with ACEA. d, rats inoculated with Aβ and i.p.-treated with SR144528. Magnification, 4×. C, quantification of the astroglial cell density as the proportional area immunostained with anti-S100B antibody, i.e., the area occupied by the positively staining cells compared with the total scanned area. Data are shown as mean ± S.E.M. of five experiments. **, $p < 0.01$ versus ctrl; °, $p < 0.05$; and °°, $p < 0.01$ versus Aβ-inoculated rats.

to attenuate Aβ pathology or to delay its onset (Mori et al., 2006).

Documented evidence herein showing that ECS modulates astroglial functioning in a complex manner reinforces the conviction that changes in endocannabinoid production and receptor expression have to be considered part of an endogenous response in which glial cells play a crucial role (Walter and Stella, 2004). A growing body of evidence now implicates that neuroprotective activity of cannabinoids occurs in a substantial measure through a series of glial-dependent anti-inflammatory actions (Fernández-Ruiz et al., 2005).

In this study, CB1 receptors were down-regulated and CB2 receptors were overexpressed in astrocytes in response to Aβ, paralleling the changes in cannabinoid receptor expression occurring in the AD brain. It was observed that the density of CB1 receptors was markedly reduced in AD patients compared with healthy subjects (Ramírez et al., 2005). Given that CB1 receptors are largely present on the SP-segregated neurons, astroglia may represent the major source of such receptors in the AD brain, so that their putative contribution to neuroprotection could be exerted primarily through astroglial cells (Ramírez et al., 2005). Our previous studies provided evidence that astroglial CB1 receptor signaling works as a molecular mechanism leading to the inhibition of proinflammatory mediators (Esposito et al., 2001). These findings, in addition to the current results, suggest that the role of CB1 receptor should no longer be limited to a straight defense process merely exerted on neuronal cells. If Aβ astroglial neurotoxicity implicates CB1 receptor down-regulation and AEA level reduction, then beside the direct protective action on neurons, agents able to stimulate these sites could represent attractive candidates for the management of pathologies where reactive gliosis is one of their hallmarks. Nevertheless, the use of CB1 receptor agonists is hampered by a variety of unwanted consequences, which include psychotropic effects, memory impairment, and mood alterations.

Conversely, it was reported that CB2 receptor expression in astrocyte-associated plaques was increased in the postmortem AD brain (Ramírez et al., 2005), even if the role of CB2 receptors in the control of astrocytes response to Aβ challenge was not clearly elucidated. Interestingly, in the model of study investigated in this study, interactions at CB2 receptors significantly modulated astroglial activation, suggesting that they possess a functioning role in the regulation of Aβ-induced reactive astrogliosis. At present, it remains unclear which signaling pathway could be specifically implicated in the generation of the dual effect induced by selective modulation of CB1 and CB2 receptors. Further investigations are required to elucidate this issue; nevertheless, the current results underline the major importance of research aimed at producing drugs able to blunt reactive astrogliosis. The modulation of astroglial cell function can be jeopardized by inflammatory events, yet it is crucial to ensure an optimal environment for neuronal survival and functioning. In conclusion, the present study interestingly demonstrates that pharmacological interactions at astroglial CB1 and CB2 receptors result in a marked and opposing regulation of Aβ-induced reactive astrogliosis. This supports the notion that future testing of selectively blocking CB2 receptor compounds may have therapeutic potential in controlling Aβ-related pathology due to their beneficial effects devoid of psychotropic consequences.

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