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Decreased endocannabinoid levels in the brain and beneficial effects of agents activating cannabinoid and/or vanilloid receptors in a rat model of multiple sclerosis

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Recent studies have addressed the changes in endocannabinoid ligands and receptors that occur in multiple sclerosis, as a way to explain the efficacy of cannabinoid compounds to alleviate spasticity, pain, tremor, and other signs of this autoimmune disease. Using Lewis rats with experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, we recently found a decrease in cannabinoid CB_1 receptors mainly circumscribed to the basal ganglia, which could be related to the motor disturbances characteristic of these rats. In the present study, using the same model, we explored the potential changes in several neurotransmitters in the basal ganglia that might be associated with the motor disturbances described in these rats, but we only found a small increase in glutamate contents in the globus pallidus. We also examined whether the motor disturbances and the changes of CB_1 receptors found in the basal ganglia of EAE rats disappear after the treatment with rolipram, an inhibitor of type IV phosphodiesterase able to supress EAE in different species. Rolipram attenuated clinical decline, reduced motor inhibition, and normalized CB_1 receptor gene expression in the basal ganglia. As a third objective, we examined whether EAE rats also exhibited changes in endocannabinoid levels as shown for CB_1 receptors. Anandamide and 2-arachidonoylglycerol levels decreased in motor related regions (striatum, midbrain) but also in other brain regions, although the pattern of changes for each endocannabinoid was different. Finally, we hypothesized that the elevation of the endocannabinoid activity, following inhibition of endocannabinoid uptake, might be beneficial in EAE rats. AM404, arvanil, and OMDM2 were effective to reduce the magnitude of the neurological impairment in EAE rats, whereas VDM11 did not produce any effect. The beneficial effects of AM404 were reversed by blocking TRPV1 receptors with capsazepine, but not by blocking CB_1 receptors with SR141716, thus indicating the involvement of endovanilloid mechanisms in these effects. However, a role for CB_1 receptors is supported by additional data showing that CP55,940 delayed EAE progression. In summary, our data suggest that reduction of endocannabinoid signaling is associated with the development of EAE in rats. We have also proved that the reduction of CB_1 receptors observed in these rats is corrected following treatment with a compound used in EAE such as rolipram. In addition, the direct or indirect activation of vanilloid or cannabinoid receptors may reduce the neurological impairment experienced by EAE rats, although the efficacy of the different compounds examined seems to be determined by their particular pharmacodynamic and pharmacokinetic characteristics.

Keywords: Cannabinoids; Endocannabinoid uptake inhibitors; CB_1 receptors; Multiple sclerosis; EAE

Introduction

Based on anecdotal data suggesting that multiple sclerosis (MS) patients self-medicate with marijuana to alleviate some of their symptoms, in particular those of a motor nature (for review, see Pertwee, 2002), recent studies have addressed the efficacy of cannabinoid-based compounds in animal models that reproduce specific pathological aspects of this autoimmune disease (Arévalo-

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Martin et al., 2003; Baker et al., 2000, 2001; for review, see Baker and Pryce, 2003). Thus, Baker et al. (2000, 2001) reported recently that plant-derived, synthetic, and endogenous cannabinoid agonists were able to reduce some signs (spasticity and tremor) in mice with chronic relapsing experimental allergic encephalomyelitis (CREAE), a mouse model of MS. They also demonstrated that these effects were mediated by cannabinoid CB_1 and, to a lesser extent, CB_2 receptors (Baker et al., 2000). Using this mouse model, they have also described anti-spastic effects of compounds that are able to inhibit the process of termination of the biological action of endocannabinoids, such as the endocannabinoid reuptake inhibitors AM404 (Baker et al., 2001), arvanil (Brooks et al., 2002), UCM707 (unpublished results), and OMDM2 (de Lago et al., 2004), and the fatty acid amide hydrolase (FAAH) inhibitor AM374 (Baker et al., 2001). These observations were concordant with the observation of increased contents of endocannabinoids in the spinal cord, and to lower extent in the brain, of these animals occurring specifically during the spastic phase of this disorder (Baker et al., 2001; see below). Using Lewis rats with experimental autoimmune encephalomyelitis (EAE), a rat model of MS generated by inoculation of guinea-pig myelin basic protein, other authors (Lyman et al., 1989; Wirguin et al., 1994) had demonstrated that a chronic administration of plant-derived cannabinoids may reduce or delay the incidence and severity of clinical signs (hind limb paralysis) in these rats.

This pharmacological evidence provided solid experimental support to the previous anecdotal, uncontrolled, or preclinical data mentioned above, which suggested a beneficial effect for marijuana when smoked by MS patients to alleviate symptoms as spasticity, dystonia, tremor, ataxia, pain, and others (for review, see Consroe et al., 1997, British Medical Association Report, 1997). Further studies demonstrated that oral Δ^9-tetrahydrocannabinol, the major component of marijuana, or nabilone, a synthetic cannabinoid, also alleviated MS symptoms (Clifford, 1983; Martyn et al., 1995; Ungerleider et al., 1987). However, in all these studies, undesired effects appeared during the course of the treatment and some authors reported an enhancement of symptomatology rather than beneficial effects (Greenberg et al., 1994). With the purpose to clarify all data in humans, a clinical trial was recently completed in the UK using oral administration of placebo, cannabis extract, or Δ^9-tetrahydrocannabinol in a population of 667 patients with stable MS and muscle spasticity. The results of this trial suggested that cannabinoids did not have a beneficial effect on spasticity in MS patients, when this is measured with the Ashworth scale, but definitively increased the patient's perception of an improvement of motor performance and pain (Zajicek et al., 2003).

Despite the progress in the pharmacological evaluation of cannabinoid-based medicines in MS both in patients and animal models, there are still no data on the possible changes in cannabinoid CB_1 and CB_2 receptors, the main molecular targets for the action of cannabinoids, in the post-mortem brain of patients with MS, while only a few studies have examined the status of the endocannabinoid transmission in animal models of this disease (Baker et al., 2001; Berrendero et al., 2001). Thus, as mentioned above, Baker and coworkers reported an increase in the levels of endocannabinoids mostly in the spinal cord of CREAE mice (Baker et al., 2001), which was interpreted by these authors as indicative of an endocannabinoid influence in the control of some symptoms of MS in an environment of existing neurological damage (see Baker and Pryce, 2003, for review). In our laboratory, using EAE rats, we recently reported a decrease of CB_1 receptor binding and mRNA levels in several brain areas (Berrendero et al., 2001), although several questions remained to be investigated, some of which have been addressed in the present study. Thus, in our previous study (Berrendero et al., 2001), we observed that the decreases in CB_1 receptors were mainly restricted to the basal ganglia (lateral and medial caudate–putamen), and to a lesser extent to cortical regions, which were related to the fact that motor deterioration is one of the most prominent neurological signs in EAE rats (Berrendero et al., 2001). Therefore, as a first objective, we explored whether EAE rats presented any potential changes in several neurotransmitters acting at the basal ganglia level, which might parallel the motor impairment presumably shown by these rats as a consequence of their neurological deficits (see McGavern et al., 1999, for details). The interest of these determinations is that cannabinoids have been reported to influence specific neurotransmitters in the basal ganglia circuitry, therefore this might be the way for cannabinoids to alleviate motor symptoms in MS patients and animal models (for review, see Fernández-Ruiz et al., 2002). Secondly, we wanted to evaluate whether the changes in CB_1 receptors found in EAE rats are closely linked to the clinical decline in these rats. To this end, we examined whether rolipram, an inhibitor of type IV phosphodiesterase used to reduce clinical impairment in EAE rodents (Folcik et al., 1999; Jung et al., 1996; Sommer et al., 1995), was able to normalize motor deterioration and the changes of CB_1 receptors in the basal ganglia. Since the previous studies dealing with the analysis of the endocannabinoid system in EAE rats only addressed the analysis of CB_1 receptors in various brain regions (Berrendero et al., 2001), as a third objective, we also explored the potential changes in endocannabinoid levels in EAE rats. Finally, based on the occurrence of reduced endocannabinoid levels in the brain of EAE rats, we hypothesized that the elevation of the endocannabinoid activity, for instance after the inhibition of the endocannabinoid transport, might be beneficial in EAE rats by reducing clinical impairment. Based on the results obtained, we have also examined the effects of direct agonists of cannabinoid CB_1 or vanilloid TRPV1 receptors, as well as the possible attenuation by antagonists of these receptors of the effects of one of the endocannabinoid uptake inhibitors, AM404, that was efficacious to delay EAE progression.

Materials and methods

Animals, treatments, and sampling

Induction of EAE

Male Lewis rats (Charles River, Madrid, Spain) were housed in a room with controlled photoperiod (08:00–20:00 light) and temperature (23 ± 1°C). They had free access to standard food and water. Animals were used at adult age (250–300 g weight) for all the experiments, which were performed according to European regulations for experimental work with animals. Rats were immunized by s.c. injection into the hind footpads with 0.1 ml of an emulsion of 25 mg of guinea pig myelin basic protein in Freund's adjuvant containing 0.5 mg of *Mycobacterium tuberculosis* (strain H37 RA, Difco, Detroit, MI, USA). Control animals were obtained by inoculation with the same emulsion without guinea pig myelin basic protein. After inoculation, rats were examined daily for the presence of neurological signs, using the following scale: 0, no clinical signs; 1, limp tail; 2, hind limb weakness; 3, severe hind limb paralysis; 4, moribundity or death

due to EAE. Grades 3 and 4 were often accompanied by urinary and fecal incontinence. Daily weight loss was also recorded. Following this method of induction of EAE, we have previously reported that first clinical signs appeared around day 9–10 after inoculation, reaching the highest neurological impairment by day 13 post-inoculation (see details in Berrendero et al., 2001; Martínez et al., 1999; Puerta et al., 2000), so that rats were used for analytical procedures by that day. To control that the changes found in previous studies (Berrendero et al., 2001), as well as in the present study, are not due to local inflammatory events occurring at the basal ganglia level, as previously published data obtained with haematoxylin–eosin staining indicated (Berrendero et al., 2001; Martínez et al., 1999; Puerta et al., 2000), we have also conducted some routinary immunohistochemical analyses of biotinylated tomato lectin, a selective microglia marker, following the procedure described by Acarin et al. (1994). The peroxidase reaction product was visualized with 3,3′ diaminobenzidine, and sections were counterstained with toluidine blue. Sections incubated in media lacking biotinylated lectin were used as negative controls.

Experimental designs

In the first experiment, EAE and control rats were used on day 13 post-inoculation for analysis of clinical score (following the above-described scale), then subjected to analysis of motor behavior in a computerized actimeter for a period of 5 min at the end of which the animals were decapitated and their brains carefully removed and rapidly frozen by immersion in 2-methylbutane cold in dry ice. All samples were stored at −80°C until processed for analysis of the contents of specific neurotransmitters in the basal ganglia. In a second experiment, EAE and control rats were treated from day 1 post-inoculation with daily s.c. injections of rolipram, kindly provided by Dr. Cedillo (Schering, Spain) at a dose of 5 mg/kg/day or vehicle (Tween 80-saline). Animals were clinically tested starting on day 9 post-inoculation up to day 13, day at which the animals were subjected to analysis of motor behavior prior to be decapitated. Their brains were removed and processed as stated for the first experiment in order to be used for analysis of CB_1 receptor mRNA levels. In a third experiment, EAE and control rats were decapitated at day 13 post-inoculation, after being subjected to clinical analysis, and their brains were removed and dissected in discrete brain structures, which were subjected to lipid extraction for analysis of endocannabinoid contents. Finally, the fourth experiment consisted of daily i.p. injections of different endocannabinoid uptake inhibitors or the vehicle (Tween 80-saline) to EAE and control rats, starting on day 9 post-inoculation up to day 12. Animals were daily examined for clinical status up to day 13. In this experiment, the following inhibitors were used: AM404 (5 mg/kg; Beltramo et al., 1997), VDM11 (5 mg/kg; De Petrocellis et al., 2000), arvanil (1 mg/kg; Melck et al., 1999), and OMDM2 (5 mg/kg; Ortar et al., 2003). In order to further explore the mechanism(s) by which these inhibitors may delay EAE progression, we conducted two additional pharmacological experiments aimed at: (i) testing the effect of two direct agonists of CB_1 and TRPV1 receptors, CP55,950 (0.1 mg/kg) and capsaicin (1 mg/kg), respectively, on EAE progression and (ii) analyzing the capability of selective antagonists of CB_1 and TRPV1 receptors, SR141716 (2 mg/kg) and capsazepine (5 mg/kg), respectively, to reverse the beneficial effect of AM404 (5 mg/kg) in delaying EAE progression. In both cases, experiments were conducted following the same experimental approach used for transporter inhibitors.

Analysis of motor behavior

Motor activity was analyzed in a computerized actimeter (Actitrack, Panlab, Barcelona, Spain). This consisted of a 45 × 45 cm arena, with surrounding infrared beams that were spaced at 2.5 cm intervals, coupled to a computerized control unit, which allows the analysis, among others, of the following parameters: (i) distance traveled in the actimeter (ambulation); (ii) mean velocity employed to run the total distance traveled; and (iii) resting time (inactivity).

Measurement of endocannabinoid contents

Procedure of extraction

Pools for each brain region, in amount ranging from 0.5 to 3 g wet weight, were extracted immediately after sacrifice to avoid the post-mortem rise in the levels of long-chain *N*-acylethanolamines (Schmid et al., 1995). The tissue was homogenized in 5 vol of chloroform/methanol/Tris–HCl 50 mM (2:1:1) containing 1 nmol of d_8-anandamide and d_8-2-arachidonoylglycerol. Deuterated standards were synthesized from d_8 arachidonic acid and ethanolamine or glycerol as described, respectively, in Devane et al. (1992) and Bisogno et al. (1997). Homogenates were centrifuged at 13,000 × *g* for 16 min (4°C), the aqueous phase plus debris were collected and extracted again twice with 1 vol of chloroform. The organic phases from the three extractions were pooled and the organic solvents evaporated in a rotating evaporator. Lyophilized samples were then stored frozen at −80°C under nitrogen atmosphere until analyzed.

Analysis of endocannabinoid contents

Lyophilized extracts were resuspended in chloroform/methanol 99:1 by vol. The solutions were then purified by open bed chromatography on silica as described in Fontana et al. (1995). Fractions eluted with chloroform/methanol 9:1 by volume (containing anandamide and 2-arachidonoylglycerol) were collected and the excess solvent evaporated with a rotating evaporator, and aliquots analyzed by isotope dilution-liquid chromatography/mass spectrometry (LC-MS) carried out under conditions described previously (Marsicano et al., 2002) and allowing the separations of 2-arachidonoylglycerol and anandamide. MS detection was carried out in the selected ion monitoring mode using *m*/*z* values of 356 and 348 (molecular ions+1 for deuterated and undeuterated anandamide), 374 and 369 (molecular ions+1 for deuterated and undeuterated 2-arachidonoylglycerol). The area ratios between signals of deuterated and undeuterated anandamide varied linearly with varying amounts of undeuterated anandamide (30 fmol–100 pmol). The same applied to the area ratios between signals of deuterated and undeuterated 2-arachidonoylglycerol in the 100 pmol–20 nmol interval. Anandamide and 2-arachidonoylglycerol levels in unknown samples were therefore calculated on the basis of their area ratios with the internal deuterated standard signal areas. Two LC-MS peaks for both deuterated and undeuterated mono-arachidonoylglycerol were found, corresponding to 2-arachidonoylglycerol and 1(3)-arachidonoylglycerol, in agreement with the previous observation that 2-arachidonoylglycerol undergoes isomerization during the purification procedure (Stella et al., 1997). Therefore, the amounts of 2-arachidonoylglycerol were calculated by adding the amounts of the two isomers. The amounts of endocannabinoids are expressed as pmol or nmol per gram of wet tissue extracted.

Autoradiographic analyses

Brain slicing

Coronal sections 20 μm-thick were cut in a cryostat, according to the Paxinos and Watson (1986) atlas. Sections were thaw-mounted onto RNAse-free gelatin/chrome alum coated slides and dried briefly at 30°C and stored at −80°C until used. Adjacent sections to those used for autoradiographic analysis were stained with cresyl-violet for the identification of the different brain nuclei. For each analysis, at least three sections per brain and a total of 4–6 brains per experimental group were processed.

Analysis of mRNA levels for CB_1 receptor by in situ hybridization

The analysis of CB_1 receptor mRNA levels was carried out according to Rubino et al. (1994). Briefly, sections were fixed in 4% paraformaldehyde for 5 min and, after rinsing twice in phosphate buffer saline, were acetylated by incubation in 0.25% acetic anhydride, prepared in 0.1 M triethanolamine/0.15 M sodium chloride (pH 8.0), for 10 min. Sections were rinsed in 0.3 M sodium chloride/0.03 M sodium citrate, pH 7.0, dehydrated, and delipidated by ethanol/chloroform series. A mixture (1:1:1) of the three 48-mer oligonucleotide probes complementary to bases 4–51, 349–396, and 952–999 of the rat CB_1 receptor cDNA (Du Pont; the specificity of the probes used was assessed by Northern Blot analysis) was 3′-end labeled with [^{35}S]-dATP using terminal deoxynucleotidyl-transferase. Sections were then hybridized with [^{35}S]-labeled oligonucleotide probes (7.5×10^5 dpm per section), washed, and exposed to X-ray film (βmax, Amersham) for 10 days and developed (D-19, Kodak) for 6 min at 20°C. The intensity of the hybridization signal was assessed by measuring the gray levels in the autoradiographic films with a computer-assisted video-densitometer. Adjacent brain sections were co-hybridized with a 100-fold excess of cold probe or with RNAse to assert the specificity of the signal.

Determinations of γ-aminobutiric acid (GABA), glutamate, serotonin, and dopamine contents by HPLC with electrochemical detection

Brains were used to manually obtain coronal slices (around 500 μm thick) at levels containing the substantia nigra, the globus pallidus, or the caudate–putamen (Palkovits and Brownstein, 1988). Subsequently, the three structures were dissected and homogenized in 20–40 vol of cold 150 mM potassium phosphate buffer, pH 6.8. Each homogenate was distributed for the analysis, using HPLC coupled to electrochemical detection, of: (i) GABA and glutamate contents, following the procedure described by Smith and Sharp (1994), (ii) serotonin and 5-hydroxyindolacetic acid (5HIAA) contents, according to the method described by Arévalo et al. (2001), and (iii) dopamine and L-3,4-dihydroxy-phenylacetic acid (DOPAC) contents, following the method described by Romero et al. (1995). An aliquot of each homogenate was used to analyze protein concentration (Lowry et al., 1951). The aliquots used for the measurement of neurotransmitter contents were diluted (1/2) with 0.4 N perchloric acid containing 0.4 mM sodium disulfite, 0.90 mM EDTA and the corresponding internal standard (*β*-aminobutiratic acid for aminoacids, *N*-methyl-serotonin for indolamines, and dihydroxybenzylamine for catecholamines). Afterwards, samples were centrifuged for 3 min ($15{,}000 \times g$) and the supernatants directly injected into the HPLC system, expect in the case of the samples for determination of GABA and glutamate contents that were subjected to a previous derivatization process with *o*-phthaldehide (OPA)–sulfite solution (14.9 mM OPA, 45.4 mM sodium sulfite, and 4.5% ethanol in 327 mM borate buffer, pH 10.4) (see details in González et al., 1999). HPLC system consisted of the following elements. The pump was an isocratic Spectra-Physics 8810. The column was an RP-18 (Spherisorb ODS-2; 150 mm, 4.6 mm, 5 μm particle size; Waters, Massachusetts, USA). The mobile phase, previously filtered and degassed, consisted of: (i) 0.06 M sodium dihydrogen phosphate, 0.06 mM EDTA, and 20–30% methanol (pH 4.4), for determination of GABA and glutamate, (ii) 100 mM citric acid, 100 mM sodium acetate, 1 mM EDTA, and 7% methanol (pH 4.2), for the determination of serotonin and 5HIAA, and (iii) 100 mM citric acid, 100 mM sodium acetate, 1.2 mM heptane sulphonate, 1 mM EDTA, and 7% methanol (pH 3.9), for the determination of dopamine and DOPAC. The flow rate was 0.8 ml/min. The effluent was monitored with a Metrohm bioanalytical system amperometric detector using a glassy carbon electrode. The potential was 0.85 V (aminoacids) or 0.80 V (indolamines) relative to an Ag/AgCl reference electrode with a sensitivity of 50 nA (approx. 2 ng per sample). In the case of the determination of dopamine and DOPAC, the effluent was monitored with a coulochemical detector (Coulochem II, ESA) using a procedure of oxidation/reduction (conditioning cell: +360 mV; analytical cell #1: +50 mV; analytical cell #2: −340 mV), procedure that reaches a sensitivity of 50 nA (10 pg per sample). In the three cases, the signal was recorded on a Spectra-Physics 4290 integrator. The results were obtained from the peaks and calculated by comparison with the area under the corresponding internal standard peak. Values were expressed as ng or μg/mg of protein.

Statistics

Data were assessed, as required, by one-way or two-way analysis of variance followed by the Student–Newman–Keuls test or by the unpaired Student's *t* test.

Results

Behavioral and neurochemical characterization of EAE rats

As reported previously (Berrendero et al., 2001; Martínez et al., 1999), EAE rats showed a pattern of progressive deterioration with the first neurological signs appearing on days 9–10 post-inoculation and reaching the highest neurological impairment by day 13, when the animals had a clinical score of grade 4 (paraplegia) (Table 1). These neurological signs are likely produced as a consequence of inflammatory events occurring mainly at the spinal level. Thus, we have previously reported, using haemotoxylin–eosin staining, that the spinal cord of EAE rats has a dense infiltration by mononuclear cells with numerous foci of perivascular inflammation, more intense in the lumbar area (Martínez et al., 1999; Puerta et al., 2000). However, infiltrates were rare in the brain and completely absent in the forebrain structures (Berrendero et al., 2001). To further confirm this, we have performed immunohistochemical analyses of tomato lectin, a marker for normal and reactive microglia, in rat brain sections obtained at the level of the caudate–putamen in EAE and control rats. Since normal and activated microglial cells can be easily differentiated by their morphology (see Acarin et al., 1994), the

Table 1
Clinical score, actimeter response, and concentrations of different neurotransmitters in the basal ganglia of EAE and control rats on day 13 post-inoculation

Parameters		Control rats	EAE rats
Clinical score		0.00 ± 0.00	3.75 ± 0.25**
Ambulation (distance traveled in cm)		798.7 ± 77.6	383.9 ± 53.5**
Mean speed (cm/s)		2.68 ± 0.26	1.29 ± 0.17**
Time spent in inactivity (s)		144.4 ± 9.3	214.9 ± 9.3**
Caudate–putamen	GABA (μg/mg protein)	2.8 ± 0.6	2.7 ± 0.2
	DA (ng/mg protein)	74.0 ± 7.2	75.2 ± 6.3
	DOPAC (ng/mg protein)	15.6 ± 2.5	14.2 ± 1.2
	Glutamate (μg/mg protein)	7.7 ± 0.2	7.2 ± 0.5
	5HT (ng/mg protein)	8.1 ± 2.9	11.0 ± 3.9
	5HIAA (ng/mg protein)	5.2 ± 1.8	5.6 ± 0.6
Globus pallidus	GABA (μg/mg protein)	4.2 ± 0.2	4.2 ± 0.4
	Glutamate (μg/mg protein)	4.1 ± 1.4	7.0 ± 0.5*
	5HT (ng/mg protein)	14.7 ± 3.6	12.0 ± 2.9
	5HIAA (ng/mg protein)	8.6 ± 2.4	8.0 ± 1.3
Substantia nigra	GABA (μg/mg protein)	6.8 ± 0.5	5.9 ± 0.5
	DA (ng/mg protein)	3.2 ± 0.7	3.3 ± 0.3
	DOPAC (ng/mg protein)	0.9 ± 0.2	1.0 ± 0.1
	Glutamate (μg/mg protein)	6.5 ± 0.3	5.8 ± 0.6
	5HT (ng/mg protein)	21.6 ± 3.9	18.8 ± 2.7
	5HIAA (ng/mg protein)	9.9 ± 1.6	8.8 ± 1.3

Details in the text. Values are means ± SEM of 4–6 determinations per group. Data were assessed by Student's t test (*$P < 0.05$; **$P < 0.005$).

comparison of immunostained sections from both groups was carried out by analyzing the morphology of positive cells using appropriate positive controls for activated microglia as a reference. According to this, we can conclude that there is not any relevant signs of activation of microglial cells in the caudate–putamen of EAE rats that might indicate the occurrence of local inflammatory events at the basal ganglia level (see Fig. 1). Therefore, it is reasonable to conclude that neurochemical modifications in forebrain structures of EAE rats, such as those found in CB_1 receptors in the basal ganglia (Berrendero et al., 2001), are possibly an adaptative response elicited by the spinal inflammatory effects leading to paralysis of hindlimbs rather than a consequence of local inflammatory events.

On the other hand, neurological deficits in EAE rats resulted in a profound impairment of motor performance. This has been shown by McGavern et al. (1999) in mice and revealed here in EAE rats by analyzing different motor parameters (distance traveled, mean velocity, time spent in inactivity) in a computerized actimeter (Table 1). Despite these motor disturbances, we were unable to reveal any significant changes in most of the specific neurotransmitters active at the basal ganglia circuitry. These included GABA, glutamate, dopamine and its metabolite, DOPAC, and serotonin and its metabolite, 5HIAA, in the caudate–putamen and the substantia nigra, and GABA, serotonin and its metabolite, 5HIAA, in the globus pallidus (Table 1). The only exception was glutamate content in the globus pallidus which exhibited a significant increase (Table 1).

Effects of rolipram on the neuropathological phenotype and the decrease of CB_1 receptors in EAE rats

We also examined whether neurological deficits, motor disturbances, and changes of CB_1 receptors in the basal ganglia found in EAE rats disappear after the treatment of these rats with rolipram, an inhibitor of type IV phosphodiesterase used to reduce clinical decline in EAE rodents (Folcik et al., 1999; Jung et al., 1996; Sommer et al., 1995). Rolipram reduced neurological impairment (Fig. 2), and, as a consequence of this improvement,

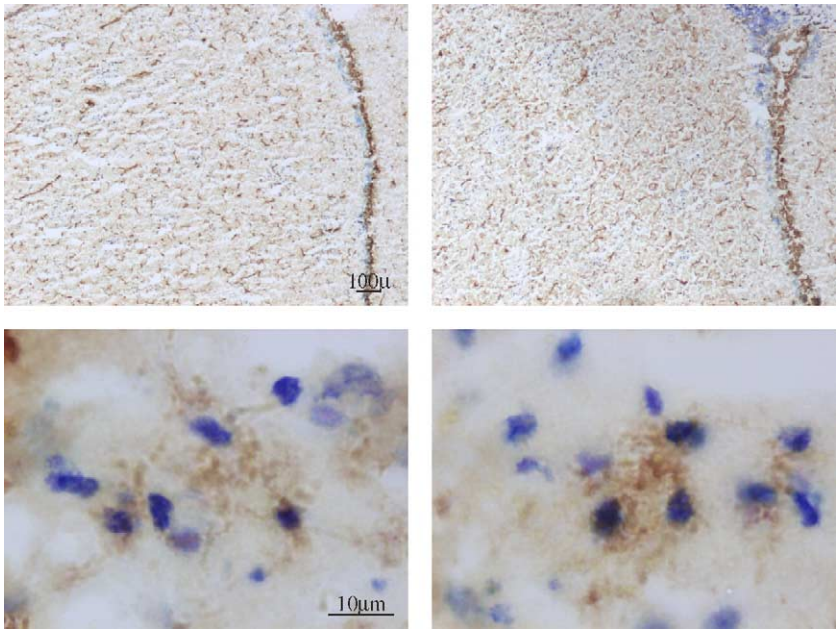

Fig. 1. Immunohistochemical identification of normal and reactive microglia by using lectin from *Lycopersicum esculentum* (tomato) in rat brain sections (at the level of the caudate–putamen) obtained from EAE and control rats on day 13 post-inoculation. Left panels are controls and right panels are EAE rats. Top panels are 4× and bottom panels are 100×.

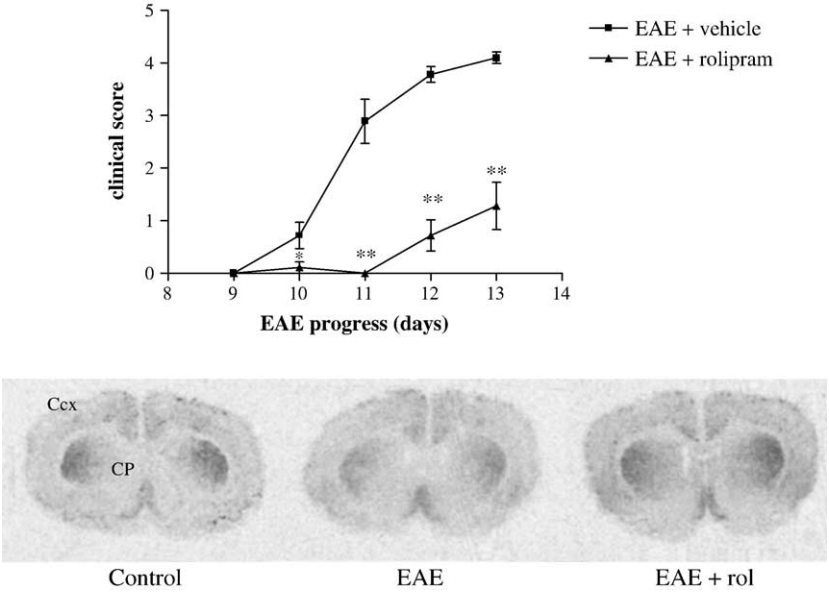

Fig. 2. Clinical decline (top panel) and autoradiograms representative of in situ hybridization analysis of CB_1 receptor mRNA levels (bottom panels; see values in text) in EAE rats subjected to daily i.p. injections of rolipram from day 9 to day 13 post-inoculation. Details in the text. Values are means ± SEM of 6–8 determinations per group. Data were assessed by two-way analysis of variance (inhibitor × time) followed by the Student–Newman–Keuls test (*$P < 0.05$, **$P < 0.005$). CP = caudate–putamen; Ccx = cerebral cortex.

it attenuated the reduction in ambulation (72.3 ± 10.6% over controls, $n = 6$) and mean velocity (71.6 ± 10.4% over controls, $n = 6$) and the increase in inactivity (131.7 ± 9.1% over controls, $n = 6$) found in EAE rats (ambulation: 48.1 ± 6.7; mean velocity: 48.1 ± 6.3; inactivity: 148.8 ± 6.4, all $P < 0.05$ vs. controls, $n = 6$). Rolipram also attenuated the decrease of CB_1 receptor mRNA levels in the lateral (87.4 ± 12.2% over controls, $n = 6$) and medial (97.5 ± 14.6% over controls, $n = 5$) caudate–putamen and the superficial (94.2 ± 12.8% over controls, $n = 4$) and deep (92.1 ± 13.6% over controls, $n = 4$) layers of the cerebral cortex found in EAE rats (lateral caudate–putamen: 75.2 ± 8.4, medial caudate–putamen: 80.0 ± 9.3; superficial layer of the cerebral cortex: 73.0 ± 10.2%; deep layer of the cerebral cortex: 80.2 ± 9.2%, all $P < 0.05$ vs. controls, $n = 4$–6) (see Fig. 2 for representative autoradiograms). Rolipram did not produce any effects in controls on the above parameters (data not shown), as well as there were no changes in CB_1 receptor mRNA levels in other brain regions, such as the hippocampus and the cerebellum (data not shown), that were not affected by EAE in our previous study (Berrendero et al., 2001).

Levels of endocannabinoids in several brain regions of EAE rats

We have also addressed the analysis of changes in endocannabinoid levels in the brain of EAE rats as shown for CB_1 receptors (Berrendero et al., 2001). We detected a marked reduction of anandamide contents in motor related regions (striatum, midbrain) and also in other regions, such as the brainstem, hippocampus, and cerebral cortex, while there were no changes in the cerebellum, diencephalon, and limbic forebrain (Fig. 3). 2-arachidonoylglycerol levels were also decreased in EAE rats, although the pattern of changes for this endocannabinoid was slightly different as compared with anandamide. Thus, 2-arachidonoylglycerol levels were also unaffected in the cerebellum and decreased in the midbrain of EAE rats but remained unchanged in the brainstem, hippocampus, striatum, and cerebral cortex (Fig. 3). By contrast, they were decreased in the diencephalon and limbic forebrain (Fig. 3).

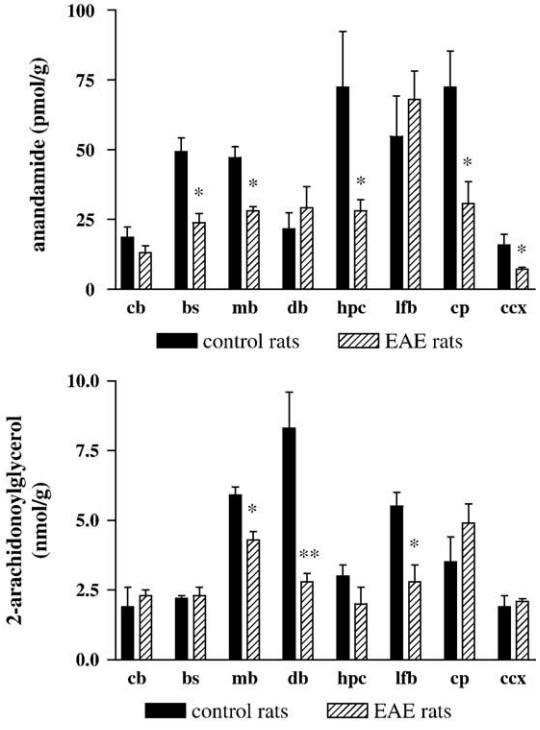

Fig. 3. Endocannabinoid contents in different brain regions of EAE and control rats on day 13 post-inoculation. Details in the text. Values are means ± SEM of 6–8 determinations per group. Data were assessed by Student's t test (*$P < 0.05$, **$P < 0.005$). Cerebellum (cb), brainstem (bs), midbrain (mb), diencephalon (db), hippocampus (hpc), limbic forebrain (lfb), caudate–putamen (cp), and cerebral cortex (ccx).

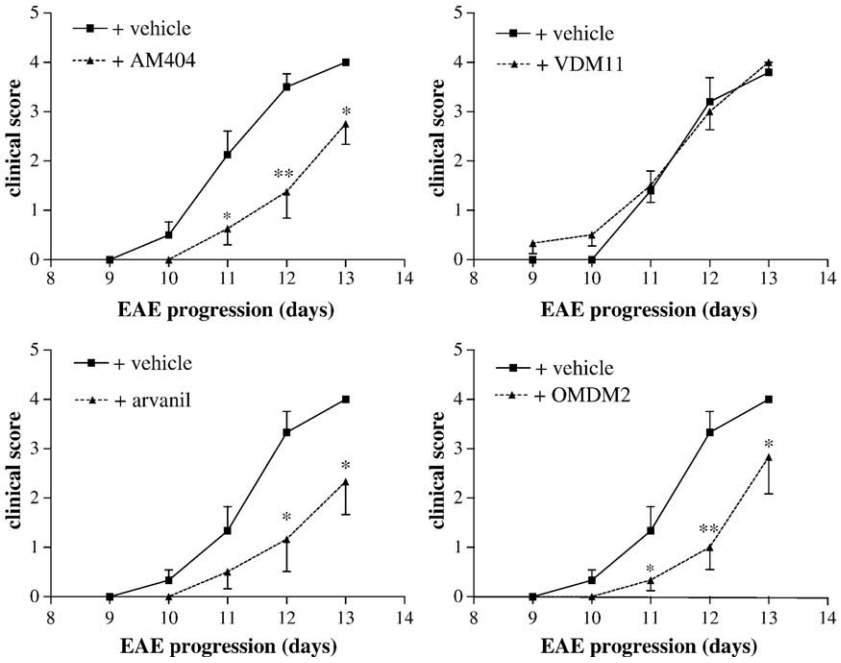

Fig. 4. Clinical decline of EAE rats subjected to daily i.p. injections of different endocannabinoid uptake inhibitors from day 9 to day 13 post-inoculation. Details in the text. Values are means ± SEM of 6–8 determinations per group. Data were assessed by two-way analysis of variance (inhibitor × time) followed by the Student–Newman–Keuls test (*$P < 0.05$, **$P < 0.01$).

Effects of "direct" or "indirect" cannabinoid or vanilloid agonists on EAE progression

Based on the fact that most of the brain regions exhibited a reduction of either anandamide or 2-arachidonoylglycerol levels in EAE rats, we hypothesized that the elevation of the endocannabinoid activity, for instance after the inhibition of the endocannabinoid transport, might be beneficial in these rats. We found that the administration of the endocannabinoid transport inhibitors and vanilloid TRPV1 receptor agonists, AM404 ($F(1,56) = 41.46$, $P < 0.0001$) and arvanil ($F(1,50) = 16.29$, $P < 0.0005$), was effective at reducing the magnitude of the neurological decline in EAE rats (Fig. 4). However, VDM11 ($F(1,45) = 1.11$, ns), which blocks the transporter with no direct effects on TRPV1 receptor, was not effective (Fig. 4), although OMDM2 ($F(1,50) = 17.38$, $P < 0.0005$), another endocannabinoid transport inhibitor which is also devoid of activity at TRPV1 receptors, did reduce neurological decline in EAE rats (Fig. 4). None of these inhibitors produced any effects on the neurological status of control rats (data not shown).

We also explored whether the beneficial effects found for AM404 in delaying EAE progression were reversed by blocking TRPV1 receptors with capsazepine or CB_1 receptors with SR141716. These two antagonists did not show cross-reaction in previous studies, so they can be used to differentiate actions mediated by TRPV1 or CB_1 receptors with high selectivity (see Di Marzo et al., 2001). Our data indicated that capsazepine was able to completely reverse AM404 effects ($F(3,95) = 6.47$, $P < 0.0005$), whereas SR141716 was not ($F(3,104) = 2.45$, ns) (Fig. 5), thus supporting the involvement of endovanilloid mechanisms in these effects. The two antagonists produced no effects when administered alone to EAE rats (data not shown). By contrast, additional data obtained by administering direct agonists of CB_1 (CP55,940) or TRPV1 (capsaicin) receptors showed that CP55,940 ($F(1,60) = 13.71$, $P < 0.0005$), but not capsaicin ($F(1,60) = 0.25$, ns), delayed EAE progression (Fig. 6), thus indicating that endocannabinoid

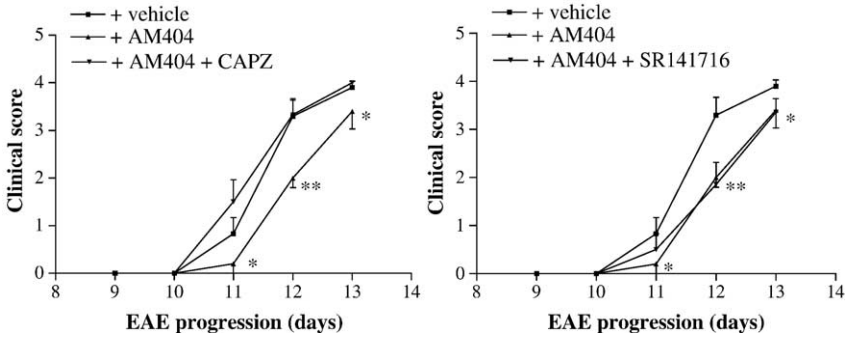

Fig. 5. Clinical decline of EAE rats subjected to daily i.p. injections of AM404 alone or in combination of either capsazepine or SR141716 from day 9 to day 13 post-inoculation. Details in the text. Values are means ± SEM of 6–8 determinations per group. Data were assessed by two-way analysis of variance (inhibitor × time) followed by the Student–Newman–Keuls test (*$P < 0.05$, **$P < 0.01$).

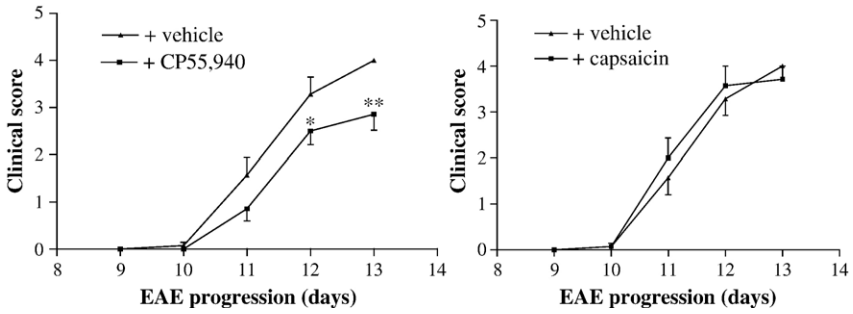

Fig. 6. Clinical decline of EAE rats subjected to daily i.p. injections of CP55,940 or capsaicin from day 9 to day 13 post-inoculation. Details in the text. Values are means ± SEM of 6–8 determinations per group. Data were assessed by two-way analysis of variance (inhibitor × time) followed by the Student–Newman–Keuls test (*$P < 0.05$, **$P < 0.01$).

mechanisms also play a beneficial role by delaying EAE progression.

Discussion

Multiple sclerosis is the neurological disease that represents the most frequent cause of chronic disability in young adults (for review, see Polman and Uitdehaag, 2000). As yet, no treatment can completely halt the accumulation of disability and substantial efforts are being made to find new therapeutic molecules in this disease. As mentioned in the Introduction, cannabinoids and related compounds have been proposed as potentially useful in the treatment of some symptoms of this disease (see references in the Introduction). However, the therapeutic potential of cannabinoids for the treatment of MS has been examined without any indication on the status of cannabinoid receptors in the post-mortem brain of patients affected by MS, except for a few and recent data in animal models of this disease (Baker et al., 2001; Berrendero et al., 2001). For instance, we recently used Lewis rats with EAE, an autoimmune inflammatory and demyelinating disease of the CNS (Martin and McFarland, 1995) that shows many similarities to human MS, to examine the status of CB_1 receptors in different brain regions (Berrendero et al., 2001). We found that changes in CB_1 receptors in EAE rats were almost uniquely restricted to regions, such as the basal ganglia (Berrendero et al., 2001), that are directly or indirectly related to the expression of MS-related signs potentially improved by cannabinoid therapy, such as tremor, dystonia, ataxia, spasticity, and others (see Baker et al., 2000). We hypothesized that these changes in CB_1 receptors might be associated with alterations at the levels of neurotransmitters acting at the basal ganglia circuitry and that the well known effects of cannabinoid agonists on these neurotransmitters might underly the improvement exerted by these compounds on motor symptoms of MS (see Fernández-Ruiz et al., 2002, for review). However, we did not find here any changes in dopamine, serotonin, GABA, or glutamate in the basal ganglia of EAE rats and for this reason we did not examine whether cannabinoid administration might influence these neurotransmitters in EAE rats. The only exception was the increase found for glutamate content in the globus pallidus of EAE rats, which, despite being a solitary event, might be related to the changes found in the endocannabinoid transmission in the basal ganglia of these rats. For instance, glutamate release in the basal ganglia is inhibited by CB_1 receptor agonists (for review, see Fernández-Ruiz et al., 2002), therefore, as EAE rats have a lower density of CB_1 receptors in the basal ganglia (Berrendero et al., 2001), an increase in glutamate content might be expected in all or some of these structures. In addition, this excess of excitatory transmission in the globus pallidus might be also a consequence of motor inhibition, probably as a compensatory or protective response, since several studies have revealed that stimulation of the external globus pallidus improves akinesia or rigidity in humans (see Yelnik et al., 2000, for review).

On the other hand, the fact that changes found for classic neurotransmitters in the basal ganglia of EAE rats are scarce, together with the observation of marked alterations in CB_1 receptors (Berrendero et al., 2001) and in their endogenous ligands (as will be discussed below), would support the notion that the changes in the endocannabinoid signaling represent the most important transmitter disturbance in the basal ganglia associated with motor deterioration of these animals. This close relationship between EAE symptoms and reduced endocannabinoid activity is also underlined by our present finding that rolipram, an inhibitor of type IV phosphodiesterase used to reduce clinical impairment in EAE, completely normalized CB_1 receptor gene expression in the basal ganglia.

As mentioned above, we have also examined in the present study whether EAE rats exhibit changes in endocannabinoid levels similar to those shown previously for CB_1 receptors (Berrendero et al., 2001). We recorded a marked reduction of both anandamide and 2-arachidonoylglycerol in motor related regions (striatum, midbrain) but also in other regions such as the brainstem, diencephalon, hippocampus, limbic forebrain, and cerebral cortex, although the pattern of changes observed for each of the two endocannabinoids analysed was different. This reduction in endocannabinoid levels is compatible with the previous data in this MS model, which showed a reduction of CB_1 receptor binding and mRNA levels, mainly in the basal ganglia (Berrendero et al., 2001), and supports the notion of a region-dependent down-regulation of endocannabinoid transmission in EAE rats. On the other hand, our present data appear to be in contrast with the data reported in the CREAE mice where an increase, albeit modest, of brain endocannabinoid levels was observed (Baker et al., 2001). It must be underlined, however, that the two models reflect very different aspects of MS pathogenesis: (i) chronic (in mice) versus acute (in rats) disease and (ii) neurodegeneration (in mice) versus inflammation (in rats).

Based on the above analytical data, one may assume that the restoration of a normal endocannabinoid tone with inhibitors of the endocannabinoid inactivation might be beneficial in EAE rats. In the present study, we have assayed several endocannabinoid

reuptake inhibitors, which present quite a few pharmacodynamic or pharmacokinetic differences. We have found that the capability to block the transporter does not appear to be the unique feature necessary to these inhibitors in order to produce beneficial effects in EAE rats. In this sense, two of these inhibitors, AM404 and arvanil, that also possess direct activity at the vanilloid TRPV1 receptors (Di Marzo et al., 2002; Zygmunt et al., 2000), were able to reduce the magnitude of the neurological impairment in EAE rats, whereas VDM11, which blocks the putative endocannabinoid transporter with no direct effects on TRPV1 receptor, was not effective. This suggested initially that direct activation of TRPV1 receptors, rather than elevation of endocannabinoid levels, might be implicated in these effects, and therefore we conducted an additional experiment addressed to examine whether selective CB_1 or TRPV1 receptor antagonists were able to reverse AM404 effects. Our data indicate that the effects of AM404 are mostly mediated by the activation of TRPV1 receptors, since they were reversed by capsazepine but not SR141716. The involvement of TRPV1 receptors is also indirectly supported by the lack of efficacy against EAE progression of UCM707 (data not shown), another inhibitor also devoid of activity at this receptor subtype (López-Rodríguez et al., 2003). However, the last transporter inhibitor examined, OMDM2, which has a pharmacodynamic profile similar to VDM11, did reduce neurological impairment in EAE rats. OMDM2 does not bind TRPV1 receptors (Ortar et al., 2003), therefore its efficacy cannot be explained by the direct activation of these receptors, suggesting that other mechanisms might be also involved, possibly related to its enhancement of endocannabinoid action at the CB_1 receptors, and to its higher metabolic stability as compared to VDM11 (Ortar et al., 2003). The involvement of CB_1 receptors is supported by previous studies (Lyman et al., 1989; Wirguin et al., 1994) showing that classic cannabinoid agonists, devoid of affinity for the TRPV1 receptor, were effective against EAE progression. In addition, Malfitano et al. (2004) recently presented preliminary evidence that arvanil delayed disease progression in EAE mice and that these beneficial effects were reversed by CB_1 receptor blockade. However, the reversion did not occur in all phases where arvanil was beneficial, thus suggesting again the involvement of more than one mechanism, perhaps the co-activation of CB_1 and TRPV1 receptors. In order to further explore the potential involvement of CB_1 receptors in delaying EAE progression, we also examined the effects of a direct agonist of this receptor subtype, CP55,940, and also of a direct agonist of TRPV1 receptors, capsaicin. Our data confirmed the involvement of CB_1 receptors, since CP55,940 was able to delay EAE progression. However, capsaicin did not exert the same effect despite the fact that data obtained with AM404 indicated that TRPV1 receptors are also involved. We believe that the differences in the properties of the compounds tested, which will be addressed below, explain the different activities observed.

In fact, the above pharmacological data, taken together, strongly suggest that the activation of either cannabinoid or vanilloid receptors might be effective to delay EAE progression. However, differences in pharmacodynamic and/or pharmacokinetic characteristics of the compound tested here, summarized in Table 2 from a number of key references, may be critical to determine their efficacy in this disease. In particular, the compounds tested possessed at least one of the following four properties: (i) capability to activate CB_1 receptors directly, as in the case of CP55,940 and arvanil; (ii) capability to activate CB_1 receptors indirectly, by inhibiting endocannabinoid uptake, as in the case of arvanil, VDM11, AM404, and OMDM2; (iii) capability to activate TRPV1 receptors, as in the case of capsaicin, AM404, and arvanil; and (iv) metabolic stability, as in the case of arvanil, OMDM2, and CP55,940. We hypothesize, from the results obtained, that at least two of these four properties must be found in the same molecule in order to observe amelioration of EAE in rats. For instance, it is possible that arvanil was already very efficacious at a dose 5-fold lower than the other compounds because it is metabolically stable and capable to activate both TRPV1 (directly) and CB_1 receptors (both directly and indirectly). By contrast, compounds that are metabolically stable, such as CP55,940 and OMDM2, may be effective even if acting only at one target. CP55,940 was efficacious because of its activity at the CB_1 receptor, and this can hold true also for OMDM2, which would act by elevating anandamide levels and potentiating their action at the CB_1 receptors. This latter effect cannot be achieved efficaciously by VDM11 (and also UCM707—data not shown), which is less metabolically stable than OMDM2 in vitro (Ortar et al., 2003) and in vivo (de Lago et al., 2004). The lack of efficacy of capsaicin may be explained by its markedly lower potency compared to arvanil at the TRPV1 receptors and its poor metabolic stability (see details and references in Table 2). Accordingly, capsaicin is two orders of magnitude less efficacious than arvanil at reducing spasticity in the CREAE mouse (Brooks et al., 2002). AM404 exhibits two of the four properties mentioned above. However, its activity against EAE in rats does not seem to be mediated by CB_1 receptors. It is possible that this compound, apart from activating TRPV1 directly, acts also

Table 2
Pharmacodynamic and pharmacokinetic characteristics of the different compound used in the present study (see McFarland and Barker, 2004; Van Der Stelt and Di Marzo, 2004, for recent reviews)

Compounds	Transporter inhibition IC50 (μM)	TRPV1 potency EC50 (nM)	Activity at the CB_1 receptor	Metabolic stability	Key references
AM404	1–5	26–35	Indirect	No	Beltramo et al. (1997), Zygmunt et al. (2000), De Petrocellis et al. (2000, 2001)
Arvanil	3.6	0.4–0.5	Indirect and direct	Yes	Melck et al. (1999), De Petrocellis et al. (2000)
VDM11	10–11	ND	Indirect	No	De Petrocellis et al. (2000)
OMDM2	3	ND	Indirect	Yes	Ortar et al. (2003)
CP55,940	ND	ND	Direct	Yes	reviewed by Pertwee (1997)
Capsaicin	ND	10–26	No	No	Park and Lee (1994), De Petrocellis et al. (2000), Di Marzo et al. (2001)

by blocking the transporter-mediated release of intracellular anandamide from TRPV1-expressing neurons [as shown for CB_1-expressing neurons (Ronesi et al., 2004)], thereby potentiating anandamide action at its intracellular binding site on TRPV1 receptors (De Petrocellis et al., 2001).

In summary, our data support the notion that a reduction of endocannabinoid signaling is associated with the expression of the neuropathological phenotype of EAE rats, particularly as both the decrease of CB_1 receptor expression observed during EAE as well as the neurological signs disappear upon treatment with rolipram. In addition, the activation of either vanilloid or cannabinoid receptors may reduce the neurological impairment experienced by EAE rats, although the efficacy of the different compounds examined seems to be determined by their particular pharmacodynamic and pharmacokinetic characteristics.

Acknowledgments

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