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Cannabinoids provide neuroprotection against 6-hydroxydopamine toxicity in vivo and in vitro: Relevance to Parkinson's disease

Isabel Lastres-Becker,[a,1] Francisco Molina-Holgado,[b] José A. Ramos,[a] Raphael Mechoulam,[c] and Javier Fernández-Ruiz[a,*]

[a]*Departamento de Bioquímica y Biología Molecular III, Facultad de Medicina, Universidad Complutense, 28040-Madrid, Spain*
[b]*Wolfson Centre for Age-Related Diseases, Division of Biomolecular Science, GKT School of Biomedical Sciences, Hodgkin Building, Guy's Campus, London SE1 1UL, UK*
[c]*Department of Medicinal Chemistry and Natural Products, Medical Faculty, Hebrew University, Jerusalem 91120, Israel*

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Cannabinoids have been reported to provide neuroprotection in acute and chronic neurodegeneration. In this study, we examined whether they are also effective against the toxicity caused by 6-hydroxydopamine, both in vivo and in vitro, which may be relevant to Parkinson's disease (PD). First, we evaluated whether the administration of cannabinoids in vivo reduces the neurodegeneration produced by a unilateral injection of 6-hydroxydopamine into the medial forebrain bundle. As expected, 2 weeks after the application of this toxin, a significant depletion of dopamine contents and a reduction of tyrosine hydroxylase activity in the lesioned striatum were noted, and were accompanied by a reduction in tyrosine hydroxylase-mRNA levels in the substantia nigra. None of these events occurred in the contralateral structures. Daily administration of Δ^9-tetrahydrocannabinol (Δ^9-THC) during these 2 weeks produced a significant waning in the magnitude of these reductions, whereas it failed to affect dopaminergic parameters in the contralateral structures. This effect of Δ^9-THC appeared to be irreversible since interruption of the daily administration of this cannabinoid after the 2-week period did not lead to the re-initiation of the 6-hydroxydopamine-induced neurodegeneration. In addition, the fact that the same neuroprotective effect was also produced by cannabidiol (CBD), another plant-derived cannabinoid with negligible affinity for cannabinoid CB_1 receptors, suggests that the antioxidant properties of both compounds, which are cannabinoid receptor-independent, might be involved in these in vivo effects, although an alternative might be that the neuroprotection exerted by both compounds might be due to their anti-inflammatory potential. As a second objective, we examined whether cannabinoids also provide neuroprotection against the in vitro toxicity of 6-hydroxydopamine. We found that the non-selective cannabinoid agonist HU-210 increased cell survival in cultures of mouse cerebellar granule cells exposed to this toxin. However, this effect was significantly lesser when the cannabinoid was directly added to neuronal cultures than when these cultures were exposed to conditioned medium obtained from mixed glial cell cultures treated with HU-210, suggesting that the cannabinoid exerted its major protective effect by regulating glial influence to neurons. In summary, our results support the view of a potential neuroprotective action of cannabinoids against the in vivo and in vitro toxicity of 6-hydroxydopamine, which might be relevant for PD. Our data indicated that these neuroprotective effects might be due, among others, to the antioxidant properties of certain plant-derived cannabinoids, or exerted through the capability of cannabinoid agonists to modulate glial function, or produced by a combination of both mechanisms.

Keywords: Cannabinoids; Parkinson's disease; 6-Hydroxydopamine; Basal ganglia; Neurodegeneration; Neuroprotection; Glial cells; Antioxidant properties; Anti-inflammatory effects

Introduction

In addition to brain functions, such as the control of nociception, motor activity, emesis, body temperature, appetite, and memory and learning, the endogenous cannabinoid signaling system has been recently implicated in the control of the cell survival/death decision in the CNS and also in the periphery (for a review, see Guzmán et al., 2001). This finding is based, among others, on the observation that cannabinoids protect neurons from toxic insults such as glutamatergic excitotoxicity (Shen and Thayer, 1998), ischemic stroke (Nagayama et al., 1999), hypoxia (Sinor et al., 2000), trauma (Panikashvili et al., 2001), oxidative stress (Hampson et al., 1998; Marsicano et al., 2002), ouabain-induced secondary excitotoxicity (van der Stelt et al., 2001a,b), and others (see recent reviews in Grundy, 2002; Grundy et al., 2001; Mechoulam et al., 2002a,b). Most of these protectant effects appear to be mediated by the activation of the cannabinoid CB_1 receptor subtype (Parmentier-Batteur et al., 2002), although the contribution

* Corresponding author. Fax: +34 91 3941691.
E-mail address: jjfr@med.ucm.es (J. Fernández-Ruiz).
[1] Present address: J.W. Goethe Universität, Section Molecular Neurogenetics, Building 26, Room 509, Theodor Stern Kai 7, 60590 Frankfurt am Main, Germany.
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of other different mechanisms (i.e., antioxidant and/or anti-inflammatory properties of cannabinoids) cannot be ruled out (see Grundy, 2002; Grundy et al., 2001; Mechoulam et al., 2002a,b).

Cannabinoids may be also neuroprotectant in Parkinson's disease (PD) (for a review, see Romero et al., 2002), a motor neurodegenerative disorder characterized by progressive death of nigrostriatal dopaminergic neurons that mainly results in bradykinesia (slowness of movement), rigidity, and tremor as major motor abnormalities (Sethi, 2002). The motor symptoms of this disorder may be significantly reduced with therapy of dopaminergic replacement, at least in the first and middle phases of the disease (Carlsson, 2002), but this does not delay/arrest the progress of neuronal injury. A possible delay/arrest has been tried with a variety of compounds that are potentially useful in acute or chronic neurodegeneration (for a review, see Vajda, 2002), such as: (i) chemical antioxidants (for a review, see Moosmann and Behl, 2002), (ii) NMDA receptor antagonists (for a review, see Alexi et al., 2000), (iii) Ca^{++} channel blockers (for a review, see Rodnitzky, 1999), and (iv) anti-inflammatory substances (for a review, see McGeer et al., 2001). However, the results obtained so far are not as promising as expected (Tintner and Jankovic, 2002). As cannabinoids share many of the above potentially neuroprotective properties (for a review, see Grundy, 2002; Grundy et al., 2001; Mechoulam et al., 2002a,b), they could be promising molecules to investigate for delaying/arresting the neuronal injury in PD, as recently reported for other motor neurodegenerative disorders, such as Huntington's disease (Lastres-Becker et al., 2004) or amyotrophic lateral sclerosis (Raman et al., 2004). In order to evaluate whether cannabinoids might provide neuroprotection also in PD, we have conducted two series of differentiated experiments addressed to demonstrate that cannabinoids were effective against the in vivo and in vitro toxicity of 6-hydroxydopamine, a toxin currently used to generate parkinsonism in laboratory animals (for a review, see Blum et al., 2001).

In the first series of experiments, we examined the ability of Δ^9-THC, or another related plant-derived cannabinoid, cannabidiol (CBD), which shares with Δ^9-THC some properties (i.e., antioxidant capability) but differs in its absence of psychotrophic effects and its negligible affinity for the cannabinoid CB_1 receptor (Pertwee, 1997), to alter in vivo the progress of neurodegeneration in rats subjected to unilateral injections into the medial forebrain bundle of 6-hydroxydopamine. Thus, Δ^9-THC or CBD was daily administered to 6-hydroxydopamine-lesioned rats as of the first day post-lesion (to ensure an action of the cannabinoid against the appearance of first signs of toxicity) and the animals were tested for the progress of neurodegeneration after 2 weeks of daily cannabinoid administration. This was evaluated by analyzing the depletion of dopamine (DA) in the striatum, as well as by analyzing mRNA levels (in the substantia nigra) and activity (caudate-putamen) of tyrosine hydroxylase (TH), the rate-limiting enzyme for DA synthesis in these neurons. These measures were done in ipsilateral structures of lesioned animals and their sham-operated controls, but also in their corresponding contralateral structures (used as an internal control to test the effects of Δ^9-THC or CBD in the absence of lesion), which allow (i) to differentiate the potential neuroprotective effects of cannabinoids (observed only in ipsilateral structures) from mere up-regulatory effects that, if occurring, would be also observed in contralateral structures, and (ii) to control the occurrence of compensatory mechanisms. Other accompanying analyses consisted of determinations of the mRNA levels of proenkephalin and substance P in the caudate-putamen, since these two peptides are selective markers for striatal-efferent neurons which (i) serve to control the specificity of the lesion (striatal-efferent neurons do not degenerate in this model), and (ii) are under the influence of nigrostriatal dopaminergic neurons (Gerfen, 1992), so they might exhibit dysfunctional effects. In addition, we also conducted a further experiment to evaluate whether termination of Δ^9-THC administration to 6-hydroxydopamine-lesioned rats after 2 weeks would result in a re-initiation of the process of neuronal injury during two subsequent weeks. This experiment also serves to control whether the potential effects of Δ^9-THC against in vivo toxicity of 6-hydroxydopamine are mainly neuroprotective (they do not disappear after discontinuation of cannabinoid treatment) or due to up-regulatory responses (they would disappear after discontinuation of cannabinoid treatment). In a parallel study, we also tested whether the lesions caused by 6-hydroxydopamine were accompanied by changes in the effectiveness of CB_1 receptors in the caudate-putamen and the substantia nigra 2 weeks after the application of the toxin. Previous studies have shown that overactivity of these receptors developed after longer periods of time (>4 weeks) following 6-hydroxydopamine application (Mailleux and Vanderhaeghen, 1993; Romero et al., 2000) as seen in other models of PD (Lastres-Becker et al., 2001). However, there are no indications that this also happens after shorter periods of time such as those used here and whether it may influence the potential neuroprotective action of cannabinoid agonists. In this additional experiment, we also analyzed the changes in mRNA levels for the vanilloid VR1 receptor subtype, which has been recently reported to be located onto nigrostriatal dopaminergic neurons (Mezey et al., 2000) that degenerate in this PD model.

As mentioned above, in addition to their antioxidant properties, cannabinoids might be neuroprotective also because of their anti-inflammatory properties, which are likely related to their ability to modulate glial influence to neurons (for a review, see Walter and Stella, 2004). This might also be important in PD since nigral cell death is accompanied by astrocyte proliferation and reactive microgliosis at the sites of neurodegeneration (McGeer et al., 2001). Even, since the cause of dopaminergic cell death in PD is still unknown, it has been postulated that alterations in glial cell function (i.e., microglial activation) may play an important role in the initiation and/or early progression of the neurodegenerative process (Chao et al., 1996; Gao et al., 2002; Hirsch et al., 1998), especially in a region like the substantia nigra which is particularly enriched in microglia and other glial cells (Kim et al., 2000). In this sense, it is well demonstrated that activated microglia produce a wide array of cytotoxic factors, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), eicosanoids, nitric oxide, and reactive oxygen species, that impact on neurons to induce neurodegeneration (Hirsch, 2000; Minghetti and Levi, 1998), and some of them have been reported to be increased in the substantia nigra and the caudate-putamen of PD patients (Mogi et al., 1994; Nagatsu et al., 2000). Based on the above findings and on the fact that cannabinoids have been reported to posses anti-inflammatory properties (Jaggar et al., 1998; Richardson et al., 1998) which may be relevant in terms of neuroprotection–i.e., cannabinoid agonists down-regulated inflammatory cytokines (TNF-α and IL-12) and up-regulated anti-inflammatory ones (IL-10) from glial cells (Smith et al., 2000)–we conducted a second series of experiments addressed to test whether the protective effects of cannabinoids against the in vivo toxicity of 6-hydroxydopamine might also be

observed in vitro and exerted by regulating glial trophic support to neurons (i.e., by increasing prosurvival factors, and/or by reducing cytotoxic ones). In these experiments, we used cerebellar granule cell cultures exposed to 6-hydroxydopamine, a model of neuronal apoptosis that some authors have used as an in vitro model to study 6-hydroxydopamine neurotoxicity relevant to PD (Daily et al., 1999; Dodel et al., 1999). Neurons were exposed to the cannabinoid agonist HU-210 either directly, by adding the cannabinoid in their culture medium, or indirectly by exposing the neuronal cultures to conditioned medium obtained from mouse mixed glial cell cultures that had been exposed to the cannabinoid agonist.

Materials and methods

Experimental design I: In vivo effects of Δ^9-THC or CBD in the progress of neurodegeneration in rats unilaterally lesioned with 6-hydroxydopamine

Animals, surgical procedures, treatments, and sampling

Animals. Male Sprague–Dawley rats (>8 weeks; approximately 250 g weight) 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. All experiments were conducted according to European rules (directive 86/609/EEC).

Unilateral injection of 6-hydroxydopamine. After pretreatment (30 min before) with desipramine (25 mg/kg, ip), and under equithesin anesthesia (3 mg/kg, ip), rats were injected stereotaxically [coordinates: −2.5 mm in reference to bregma, −1.8 mm from the midline, −8.9 mm ventral from the dura mater, according to Paxinos atlas (Paxinos and Watson, 1986)] into the medial forebrain bundle with 6-hydroxydopamine free base (8 μg in a volume of 2 μl of saline containing 0.05% ascorbate to avoid oxidation). The correct location of this stereotaxic injection was routinely checked in a few additional animals subjected to injections of black ink and further inspection of their brains (see details in Romero et al., 2000). This was also checked at the time that rat brains were sliced for in situ hybridization analyses. Those animals showing an incorrect location of the lesion were discarded. To control the damage produced by the stereotaxic surgery itself, control rats were subjected to sham-operation (without injecting the toxin) in the ipsilateral side, whereas, in all groups, contralateral structures were always intact, allowing to measure the effects of the administered substances in the absence of lesion (contralateral structures), or after lesion or sham-operation (ipsilateral structures).

Treatment with Δ^9-THC and CBD. Δ^9-THC was kindly provided by GW Pharmaceuticals Ltd (Salisbury, UK) and CBD was purified from hashish in the Hebrew University laboratory as previously described (Gaoni and Mechoulam, 1971). They were prepared in Tween 80–saline solution (1:16 v/v) for ip administration. The doses used for each experiment were selected from previous studies reporting protective effects of these compounds in equivalent injury models (see Grundy, 2002; Grundy et al., 2001; Mechoulam et al., 2002a,b). In separate experiments, 6-hydroxydopamine-injected animals were ip administered with either Δ^9-THC (3 mg/kg weight) or CBD (3 mg/kg weight), and with their corresponding vehicles, 16 h after the local injection of 6-hydroxydopamine. The injections were repeated daily for a period of 2 weeks post-lesion, when the animals were killed 2 h after the last injection. Their brains were rapidly removed and frozen in 2-methylbutane cooled in dry ice, and stored at −80°C for neurochemical evaluation indicative of the degree of 6-hydroxydopamine-induced neuronal injury. In an additional experiment, 6-hydroxydopamine-injected rats were daily injected, starting at 16 h post-lesion, with Δ^9-THC (3 mg/kg weight) or vehicle during a period of 2 weeks. Then, the treatment was interrupted for an additional period of 2 weeks at the end of which, the animals were killed and their brains removed and processed as described for the above experiments.

Neurochemical evaluation of neuronal injury

Dissection procedure. Coronal slices (around 500 μm thick) were manually obtained at the caudate-putamen level (Palkovits and Brownstein, 1988). Subsequently, this structure was dissected and homogenized in 40 vol of cold 150 mM potassium phosphate buffer, pH 6.8. Each homogenate was distributed for the analysis of DA and DOPAC contents, and of TH activity described below.

Analysis of DA and DOPAC contents. The contents of DA and its major intraneuronal metabolite, DOPAC, were analyzed using HPLC with electrochemical detection according to our previously published method (González et al., 1999; Romero et al., 1995). Briefly, homogenates were diluted (1/2) in ice-cold 0.4 N perchloric acid containing 0.4 mM sodium disulfite and 0.90 mM EDTA. Dihydroxybenzylamine was added as an internal standard. The diluted homogenates were then centrifuged and the supernatants injected into the HPLC system, which consisted of a Spectra-Physics 8810 isocratic pump. The column was a RP-18 (Spherisorb ODS-2; 125 mm, 4.6 mm, 5 μm particle size; Waters, Massachusetts, USA). The mobile phase consisted of 100 mM citric acid, 100 mM sodium acetate, 1.2 mM heptane sulphonate, 1 mM EDTA, and 7% methanol (pH 3.9), and the flow rate was 0.8 ml/min. 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). The signal was recorded from analytical cell #2, with a sensitivity of 50 nA (10 pg per sample), on a Spectra-Physics 4290 integrator, and the results were given as area under the peaks. Values were expressed as ng/area.

Assay of TH activity. The activity of this enzyme was measured according to Nagatsu et al. (1979). Homogenates were incubated at 37°C in the presence of 0.1 M sodium acetate, 1 mM 6-methyl-5,6,7,8-tetrahydropterine (prepared in 1 M mercapto-ethanol solution), 0.1 mg/ml catalase, and 0.2 mM L-tyrosine. For the blank incubation, L-tyrosine was replaced by D-tyrosine. Blank tubes containing 1 μM L-3,4-dihydroxyphenylalanine (L-dopa) were also used as an internal standard for each tissue. After 30 min of incubation, the reaction was stopped by the addition of 0.2 N perchloric acid containing 0.2 mM sodium disulfite and 0.45 mM EDTA. Dihydroxybenzylamine was also added as an internal standard for HPLC determination. The amounts of L-dopa formed were evaluated by HPLC following the same procedure as for the direct analysis of DA and DOPAC contents, with the only difference of a previous extraction with alumina. Values were expressed as ng of L-dopa formed/area h.

Autoradiography and in situ hybridization techniques

Brain slicing. Coronal sections, 20-μm-thick, were cut in a cryostat, according to the Paxinos and Watson atlas (1986). 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.

Autoradiography of cannabinoid receptor binding. The protocol used is basically the method described by Herkenham et al. (1991). Briefly, slide-mounted brain sections were incubated for 2.5 h, at 37°C, in a buffer containing 50 mM TRIS with 5% bovine serum albumin (fatty acid-free), pH 7.4, and 10 nM [^{3}H]-CP-55,940 (Du Pont NEN) prepared in the same buffer, in the absence or the presence of 10 μM non-labeled CP-55,940 (kindly supplied by Pfizer) to determine the total and the non-specific binding, respectively. Following this incubation, slides were washed in 50 mM TRIS buffer with 1% bovine serum albumin (fatty acid-free), pH 7.4, for 4 h (2 × 2 h) at 0°C, dipped in ice-cold distilled water, and then dried under a stream of cool dried air. Autoradiograms were generated by apposing the labeled tissues, together with autoradiographic standards ([^{3}H] micro-scales, Amersham), to tritium-sensitive film ([^{3}H]-Hyperfilm MP, Amersham) for a period of 2 weeks. An intensifying screen (Biomax Transcreen LE, Kodak) was also used. Autoradiograms were developed (D-19, Kodak) for 4 min at 20°C, and the films were analyzed and quantitated in a computer-assisted videodensitometer using the standard curve generated from [^{3}H]-standards.

Analysis of mRNA levels for CB_1 receptor, VR1 receptor, TH, proenkephalin, and substance P 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 1 week, and developed (D-19, Kodak) for 6 min at 20°C. The intensity of the hybridization signal was assessed by measuring the grey levels in the autoradiographic films with a computer-assisted videodensitometer. Adjacent brain sections were co-hybridized with a 100-fold excess of cold probe or with RNAse to assert the specificity of the signal (data not shown). Similar procedures were used for the analysis of mRNA levels of proenkephalin, substance P, vanilloid VR1 receptor, and TH. We used commercial probes (NEN-Du Pont, Itisa, Madrid, Spain) for TH (García-Gil et al., 1998) and proenkephalin (Young et al., 1986), a synthetic 45-base probe, selected from the previously-published sequence, for substance P (5′-CGTTTGCCCAT-CAATCCAAAGAACTGCTGAGGCTTGGGTCTCCG-3′; Nawa et al., 1984), and a cDNA kindly provided by Dr. David Julius (University of California, San Francisco, CA, USA) for VR1 receptor (Mezey et al., 2000). Details on these procedures have been already published (Lastres-Becker et al., 2002).

Experimental design II: Effects of HU-210 on neuronal death induced by 6-hydroxydopamine in cultured cerebellar granule neurons

Cell culture, treatments, and sampling

Animals. One-day-old C57BL/6 mice were obtained from Charles River (UK) and were used for experimental purposes in accordance with the guidelines set by the European Council directives (86/609/EEC) and the Home Office, Animals Scientific Procedures Act (1986, UK).

Primary mixed glial cultures. Primary mixed glial cultures were prepared from the whole brains of 1-day-old mice following well-established protocols (McCarthy and de Vellis, 1980; Molina-Holgado et al., 1995), and grown in T150 flasks for at least 14 days in Dulbecco's modified Eagle's medium (DMEM) and 10% heat-inactivated fetal bovine serum (FBS), 20 mM glutamine, and antibiotics (0.1 IU/ml penicillin, 0.1 μg/ml streptomycin solution). The medium was changed twice per week. On reaching the confluence (usually at 2 weeks), the cells were trypsinized. The media were replaced and the cells (5 × 10^5 cells/well) were allowed to recover for 2–3 days before the experiments. To visualize glial fibrillary acidic protein (GFAP) and CD11b (MAC-1 a^M chain), the cells were washed three times with phosphate-buffered saline (PBS) at room temperature. Monoclonal antibodies to GFAP (1:500; Sigma-Aldrich Co., UK) and MAC-1 (1:100; Serotec Ltd, UK) were diluted in DMEM containing 5% FBS, 0.02% sodium azide, 0.2% bovine serum albumin (BSA), 5% goat serum, and 0.2% Triton X-100, and applied for 15 min at room temperature. Afterwards, the cells were washed three times with PBS at room temperature. The second antibodies Texas-red, conjugated donkey anti-mouse, and FITC goat conjugated anti-rat (Jackson ImmunoResearch, USA) were diluted (1:100) and applied under the same conditions. Cells were then fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Cell nuclei were labeled with DAPI (present in the mounting medium) (Vectashield, Vector, Burlingame, CA). The resulting cultures consisted of 70% astrocytes as determined by staining with GFAP and 30% of cells were positive for the microglia marker, MAC-1 (not shown).

Cerebellar granular neuronal cultures. Primary cultures of cerebellar granule neurons were prepared from the cerebella of 7-day-old mice according to well-established protocols (Cambray-Deakin, 1995). In brief, cerebella were removed and cultured in basal Eagle's medium (BME), supplemented with 10% heat-inactivated fetal calf serum (FCS), 30 mM glucose, 2 mM glutamine, and antibiotics (0.1 IU/ml penicillin, 0.1 μg/ml streptomycin solution) and 25 mM KCl. Cells were plated onto poly-lysine-coated Petri dishes, multiwells, or glass coverslips according to experimental requirements at a density of 2.5 × 10^5 cells/cm^2. To prevent glial cell proliferation, 20 h after plating, cultures were treated with cytosine-β-D-arabinofuranoside at a final concentration of 10 μM. These cultures were used at 7 days after plating, when the cell population comprises 95% granule neurons and 5% of other cell types including astrocytes (not shown).

Cell treatments. In a first experiment, primary cultures of cerebellar granule neurons were incubated for 24 h with two doses of the synthetic and non-selective cannabinoid agonist HU-210 (1 or 10 μM) (Mechoulam et al., 1990) or 6-hydroxydopamine (20 μM). HU-210 is chemically related to classic cannabinoids, but it is much more potent than Δ^9-THC or CBD at the two cannabinoid receptor subtypes, thus allowing to be used at lower concentrations in vitro and solving the solubility problems of Δ^9-THC or CBD in aqueous solutions. In a second experiment, HU-210 (1 or 10 μM) was first added to primary cultures of mixed glial cells, then incubated for 24 h, and their media removed and added to primary cultures of cerebellar granule neurons together with 6-hydroxydopamine (20 μM), and incubated for another 24 h. The above concentrations and times of incubation were determined according to previously reported experiments in glial or neuronal cultures (Dodel et al., 1999; Galea et al., 1992; Simmons and Murphy, 1992; Molina-Holgado et al., 2003). The cells were checked for their viability and proliferation, using Trypan blue dye exclusion and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays (Carmichael et al., 1987). Glial cell treatments were performed at the same density (5×10^5 cells/well, 12-well dishes).

Analysis of neuronal survival. Hoechst 33342 (10 μM) and propidium iodide (10 μM) were used to stain viable and dead cells, respectively. Cells were counted by using a fluorescence microscope (Nikon EFD3). Five to ten microscopic fields were counted for each coverslip, and two to three coverslips per treatment were used for each experiment. Only neurons positive to Hoechst 33342, but negative to propidium iodide, were counted for the final analysis of cell survival.

Statistics

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

Results

Experimental design I: In vivo effects of Δ^9-THC or CBD in the progress of neurodegeneration in rats unilaterally lesioned with 6-hydroxydopamine

Status of CB_1 receptors in the basal ganglia of 6-hydroxydopamine-injected rats

Previous studies have revealed that 6-hydroxydopamine-induced lesions up-regulate CB_1 receptors in the basal ganglia (Mailleux and Vanderhaeghen, 1993; Romero et al., 2000), but this occurred after longer periods of time after the lesion than those used by us, namely when the dopaminergic injury is expected to be high. In the present study, however, we used a shorter period for the 6-hydroxydopamine action that likely causes a moderate lesion, which mimics that found in the first phases of PD in humans, possibly the most sensitive period during which the protective effects of cannabinoids may be more significant. Therefore, it was interesting to analyze the status of CB_1 receptors in the basal ganglia (and in other reference structures), before examining the neuroprotective effects of Δ^9-THC and CBD in this rat model of PD. Our results indicated a complete lack of changes in both binding capacity and mRNA levels for CB_1 receptors 2 weeks post-lesion in the caudate-putamen (medial and lateral parts) and also in the cerebral cortex (deep and superficial layers) (see Table 1). The same lack of changes for CB_1 receptor binding occurred in the substantia nigra (Table 1), although this structure showed a small but statistically significant reduction in mRNA levels for vanilloid VR1 receptors (Table 1) since this receptor subtype has been recently reported to be located on nigrostriatal dopaminergic neurons that degenerate by the application of 6-hydroxydopamine (Mezey et al., 2000). All the above data were seen by comparing both (i) the lesioned side versus the non-lesioned side in 6-hydroxydopamine-injected rats (data not shown), and (ii) the lesioned side in 6-hydroxydopamine-injected rats versus the equivalent side in control (sham-operated) rats (see values in Table 1).

Effects of a chronic administration of Δ^9-THC to 6-hydroxydopamine-injected rats

As expected, 6-hydroxydopamine injection produced, 2 weeks post-injection, a significant depletion of DA (−46.3%; $F(2,29) = 4.323$, $P < 0.05$) and DOPAC (−35.2%; $F(2,29) = 3.70$, $P < 0.05$) contents and a reduction of TH activity (−47.3%; $F(2,29) = 9.473$, $P < 0.005$) in the striatum of the lesioned side compared with the ipsilateral structure in sham-operated animals (see values in Table 2). There was also a reduction, to a lesser extent, in TH-mRNA levels in the substantia nigra (−19.9%; $F(2,23) = 6.622$, $P < 0.01$) (Table 2). None of these events occurred in the contralateral structures (all intact) for DA (controls: 79.4 ± 8.7 ng/area; 6-hydroxydopamine: 74.1 ± 6.8), DOPAC (controls: 8.2 ± 0.8 ng/area; 6-hydroxydopamine: 7.4 ± 0.9), TH activity (controls: 242.3 ± 24.0 ng/area h;

Table 1
Cannabinoid CB_1 receptor binding (fmol/mg of protein) and mRNA levels (optical density), and vanilloid VR1 receptor mRNA levels (optical density), in the basal ganglia and some reference structures (cerebral cortex) of rats with unilateral lesions of the nigrostriatal dopaminergic neurons caused by local injection of 6-hydroxydopamine (2 weeks post-lesion) or controls (sham-operated)

Brain regions	Parameter	Control rats	6-Hydroxydopamine-lesioned rats
Lateral caudate-putamen	CB_1 receptor binding	71.5 ± 5.1	69.6 ± 3.9
	CB_1 receptor mRNA levels	0.238 ± 0.026	0.262 ± 0.019
Medial caudate-putamen	CB_1 receptor binding	55.8 ± 4.4	59.0 ± 3.3
	CB_1 receptor mRNA levels	0.143 ± 0.025	0.157 ± 0.015
Substantia nigra	CB_1 receptor binding	177.7 ± 10.5	178.5 ± 9.9
	VR1 receptor mRNA levels	0.62 ± 0.07	0.46 ± 0.06*
Cerebral cortex (deep layer)	CB_1 receptor binding	50.1 ± 3.0	52.7 ± 2.8
	CB_1 receptor mRNA levels	0.129 ± 0.027	0.144 ± 0.014
Cerebral cortex (superficial layer)	CB_1 receptor binding	40.0 ± 2.4	37.5 ± 3.2
	CB_1 receptor mRNA levels	0.112 ± 0.026	0.127 ± 0.012

Details in the text. Values are expressed as means ± SEM of at least 7 determinations per group. Data were assessed by the Student's *t* test (*$P < 0.05$).

Table 2
Effects of 2 weeks of daily administration of Δ^9-THC (3 mg/kg) or CBD (3 mg/kg), or their corresponding vehicle, on dopamine and DOPAC contents, tyrosine hydroxylase (TH) activity, and mRNA levels for proenkephalin (PENK) and substance P (SP) in the caudate-putamen, and TH-mRNA levels in the substantia nigra of rats with unilateral lesions of the nigrostriatal dopaminergic neurons caused by local injection of 6-hydroxydopamine or controls (sham-operated)

Parameters	Controls	6-hydroxydopamine-lesioned rats	
		+vehicle	+Δ^9-THC
Caudate-putamen:			
Dopamine contents (ng/area)	67.4 ± 10.5	36.2 ± 6.4*	48.2 ± 6.4
DOPAC contents (ng/area)	5.85 ± 1.16	3.79 ± 0.65*	5.32 ± 0.70
TH activity (ng/area.h)	237.6 ± 23.6	125.1 ± 15.3***	194.0 ± 19.2#
PENK-mRNA levels (optical density)	0.105 ± 0.023	0.112 ± 0.020	0.137 ± 0.012
SP-mRNA levels (optical density)	0.128 ± 0.004	0.121 ± 0.009	0.147 ± 0.011
Substantia nigra:			
TH-mRNA levels (optical density)	0.381 ± 0.009	0.305 ± 0.021**	0.401 ± 0.026#

Parameters	Controls	6-hydroxydopamine-lesioned rats	
		+vehicle	+CBD
Caudate-putamen:			
Dopamine contents (ng/area)	81.9 ± 7.8	52.2 ± 6.9**	70.0 ± 3.5#
DOPAC contents (ng/area)	7.50 ± 1.29	5.59 ± 0.60	8.02 ± 1.09
TH activity (ng/area.h)	222.6 ± 26.3	127.9 ± 13.8**	196.0 ± 13.4#
PENK-mRNA levels (optical density)	0.111 ± 0.007	0.113 ± 0.005	0.104 ± 0.009
SP-mRNA levels (optical density)	0.046 ± 0.005	0.043 ± 0.003	0.039 ± 0.003
Substantia nigra:			
TH-mRNA levels (optical density)	0.199 ± 0.023	0.117 ± 0.021*	0.149 ± 0.016

Data correspond to values measured in ipsilateral structures in three experimental groups, while the values in contralateral structures are included in the text. Values are expressed as means ± SEM of at least 7 determinations per group. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ versus control rats; #$P < 0.05$ versus vehicle-injected 6-hydroxydopamine-lesioned rats).

6-hydroxydopamine: 231.2 ± 17.5), and TH-mRNA levels (controls: 0.374 ± 0.009 units of OD; 6-hydroxydopamine: 0.401 ± 0.019). Daily administration of Δ^9-THC (3 mg/kg) during 2 weeks after the lesion produced a significant waning in the magnitude of the above reductions caused by the toxin in DA and DOPAC contents, and TH activity and mRNA levels, comparing the ipsilateral structures of the three experimental groups (see Table 2). No changes occurred in the contralateral non-lesioned structures by the exposure to Δ^9-THC (DA: 78.9 ± 9.8 ng/area; DOPAC: 7.6 ± 1.0 ng/area; TH activity: 229.0 ± 27.3 ng/area h; TH-mRNA levels: 0.435 ± 0.014 units of OD). By contrast, the mRNA levels of proenkephalin and substance P in the caudate-putamen were not altered by either administration of 6-hydroxydopamine alone or when animals were also ip injected with Δ^9-THC (Table 2).

Effects of chronic administration of CBD to 6-hydroxydopamine-injected rats

We next studied whether the above neuroprotective actions of Δ^9-THC were also exerted by CBD, a cannabinoid also derived from *Cannabis sativa*, which shares with Δ^9-THC some properties (i.e., antioxidant capability) but differs in its lack of affinity for the CB_1 receptors (for a review, see Bisogno et al., 2001; Pertwee, 1997). Also, in the animals of this experiment, 6-hydroxydopamine injection reduced, 2 weeks post-injection, DA (−36.3%; $F(2,29) = 6.147$, $P < 0.01$) contents and TH activity (−42.5%; $F(2,29) = 7.766$, $P < 0.005$) in the caudate-putamen, and TH-mRNA levels (−41.2%; $F(2,29) = 4.767$, $P < 0.05$) in the substantia nigra, whereas the reduction in DOPAC content in the caudate-putamen did not reach statistical significance in this experiment (see values in Table 2). As in the above experiment, these reductions were observed by comparing the ipsilateral structures of 6-hydroxydopamine-injected and sham-operated animals, whereas none of these events occurred in the contralateral structures for DA (controls: 102.0 ± 9.1 ng/area; 6-hydroxydopamine: 92.6 ± 7.1), DOPAC (controls: 10.4 ± 2.1 ng/area; 6-hydroxydopamine: 8.1 ± 0.8), TH activity (controls: 229.0 ± 19.9 ng/area h; 6-hydroxydopamine: 247.9 ± 19.3), and TH-mRNA levels (controls: 0.154 ± 0.028 units of OD; 6-hydroxydopamine: 0.146 ± 0.018). It is important to note that, in general, slightly different values were recorded for some of these parameters in this and the above experiment (see Table 2), differences that may be attributed to a normal interassay variation due to factors such as small differences in weight and age of animals or seasonal variations. Daily administration of CBD (3 mg/kg), during these 2 weeks post-lesion, also produced a significant waning in the magnitude of the above reductions caused by the toxin in DA and DOPAC contents and TH activity and mRNA levels, also causing a complete recovery of the control values in some cases (see Table 2). As occurred with Δ^9-THC, the effects of CBD were observed comparing the ipsilateral structures of the three experimental groups, but they did not occur in the contralateral non-lesioned structures (DA: 107.6 ± 8.6 ng/area; DOPAC: 10.6 ± 0.8 ng/area; TH activity: 285.9 ± 23.5 ng/area h; TH-mRNA levels: 0.144 ± 0.016 units of OD). In addition, they were not accompanied by changes in mRNA levels of proenkephalin and substance P in the caudate-putamen in any of the three experimental groups analyzed (Table 2).

Effects of the interruption in the chronic administration of Δ^9-THC to 6-hydroxydopamine-injected rats

A further objective of our study was to examine whether 2 weeks after the end of the chronic Δ^9-THC administration to 6-hydroxydopamine-lesioned rats, a re-initiation of the process of neuronal injury would take place. Our results indicated that the protective effect of Δ^9-THC appeared to be irreversible since, 2

weeks after the interruption of this treatment, there were still statistical differences between the ipsilateral structures of Δ^9-THC- and vehicle-treated 6-hydroxydopamine-injected rats as regards to DA ($F(2,20) = 4.035$, $P < 0.05$) and DOPAC ($F(2,20) = 7.411$, $P < 0.005$) contents and TH activity ($F(2,20) = 4.49$, $P < 0.05$) in the caudate-putamen, and to mRNA levels for TH ($F(2,20) = 6.382$, $P < 0.01$) in the substantia nigra (see Fig. 1). Again, no changes were noted in the contralateral non-lesioned structures for DA (+vehicle: 50.2 ± 7.3 ng/area; +Δ^9-THC: 51.5 ± 5.2), DOPAC (+vehicle: 6.8 ± 0.4 ng/area; +Δ^9-THC: 5.5 ± 0.8), TH activity (+vehicle: 120.5 ± 11.6 ng/area h; +Δ^9-THC: 132.6 ± 28.4), and TH-mRNA levels (+vehicle: 0.098 ± 0.004 units of OD; +Δ^9-THC: 0.102 ± 0.007), whereas mRNA levels for proenkephalin and substance P in the caudate-putamen were not altered after injection of Δ^9-THC or vehicle to 6-hydroxydopamine-lesioned animals (Fig. 1).

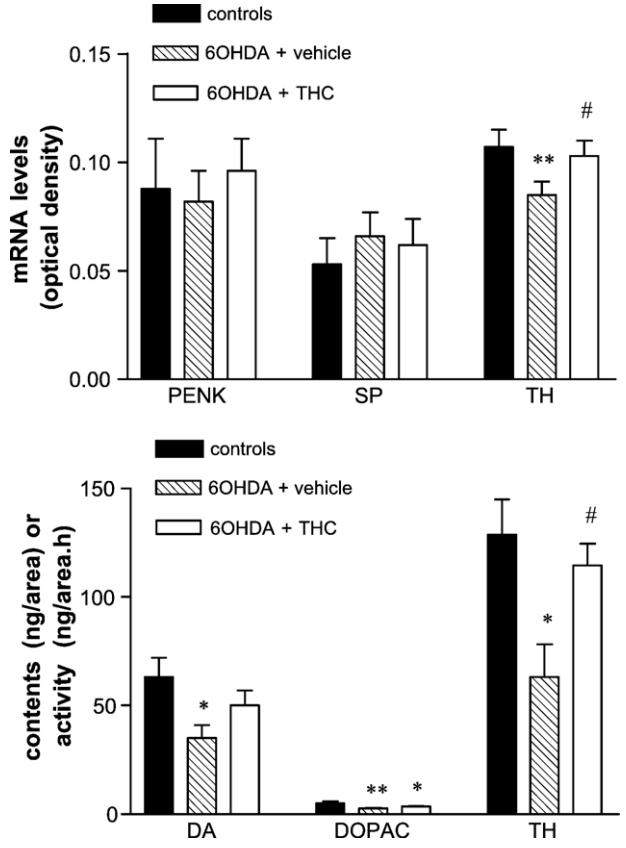

Fig. 1. Effects of 2 weeks of daily administration of Δ^9-THC (3 mg/kg) or vehicle, followed by a period of another 2 weeks in which the treatment was interrupted, on dopamine and DOPAC contents, tyrosine hydroxylase (TH) activity, and mRNA levels for proenkephalin (PENK) and substance P (SP) in the caudate-putamen, and TH-mRNA levels in the substantia nigra of rats with unilateral lesions of the nigrostriatal dopaminergic neurons caused by local injection of 6-hydroxydopamine or controls (sham-operated). Data correspond to values measured in ipsilateral structures in the three experimental groups, while the values in contralateral structures are included in the text. Values are expressed as means ± SEM of at least 8 determinations per group. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test (*$P < 0.05$, **$P < 0.01$ versus control rats; #$P < 0.05$ versus vehicle-injected 6-hydroxydopamine-lesioned rats).

Experimental design II: Effects of HU-210 on neuronal death induced by 6-hydroxydopamine in cultured cerebellar granule neurons

To assess the neuroprotective effect of cannabinoid agonists on 6-hydroxydopamine-induced neuronal death in vitro, we used mouse cultures of cerebellar granule cells. These cells are quite sensitive to 6-hydroxydopamine, so that they have been used as an in vitro model to test the neurotoxicity of this toxin which may be relevant to PD (Daily et al., 1999; Dodel et al., 1999; Offen et al., 2000). We observed that the addition of 6-hydroxydopamine to differentiated cerebellar granule neurons during a period of 24 h caused a dramatic reduction in the number of surviving cells (Fig. 2), similar to that found by other authors (Kumar et al., 1995; Lotharius et al., 1999). Neuronal death developed rapidly, and the number of viable cells was reduced approximately to 35% of the total number of cerebellar granule neurons in culture (neuronal survival at control group was considered as 100%). Interestingly, the exposure of these neurons to the non-selective agonist HU-210, a cannabinoid much more better for in vitro studies than plant-derived cannabinoids, reduced 6-hydroxydopamine-induced cell death ($F(5,35) = 41.59$, $P < 0.0001$; Fig. 2), but this effect, compared with the effect of cannabinoids observed in the in vivo experiments, was small and did not exhibit dose-dependency (neuronal survival with HU-210 1 μM: 55%, and with HU-210 10 μM: 49%). It is possible that this might be related to the fact that some neuroprotective substances act in vivo by increasing prosurvival glial influence to neurons, which cannot be reproduced with this experimental approach. To solve this, we used the experimental design described by De Bernardo et al. (2003), who demonstrated that conditioned media obtained from glial cell cultures may increase neuronal survival in vitro. Thus, cerebellar granule neuronal cultures were treated with 6-hydroxydopamine and conditioned medium obtained by exposure of mixed glial cell cultures to HU-210 1 or 10 μM, also for 24 h. We observed that, compared with the small effect when HU-210 is directly added to cultured neurons, the neuronal survival rate was quite increased when exposure to this cannabinoid was indirect (through generating glial conditioned media) (see Fig. 2). This suggests that the neuroprotective effect of HU-210 could be mainly exerted by increasing prosurvival glial influence to neurons. In addition, the effect showed a good dose-dependency (neuronal survival with HU-210 1 μM: 44%, and 10 μM: 88%, see Fig. 2), which might be indicative of the involvement of cannabinoid receptors, either CB_1 or CB_2, or both, in these effects.

Discussion

The present study shows the first evidence for a neuroprotective action of cannabinoids in an animal model of PD, an adult-onset neurodegenerative disorder characterized by a preferential loss of the dopaminergic neurons of the substantia nigra pars compacta (for a review, see Sethi, 2002) triggered by three major pathogenic events: oxidative stress, mitochondrial dysfunction, and inflammatory stimuli (McGeer et al., 2001; Sherer et al., 2001). Previous studies relating cannabinoids to PD addressed questions as the changes in the endocannabinoid signaling system in postmortem basal ganglia of PD patients (Hurley et al., 2003; Lastres-Becker et al., 2001) or in animal models of this disease (Di Marzo et al., 2000; Gubellini et al., 2002; Lastres-Becker et al., 2001; Romero et al., 2000; Silverdale et al., 2001; Zeng et al.,

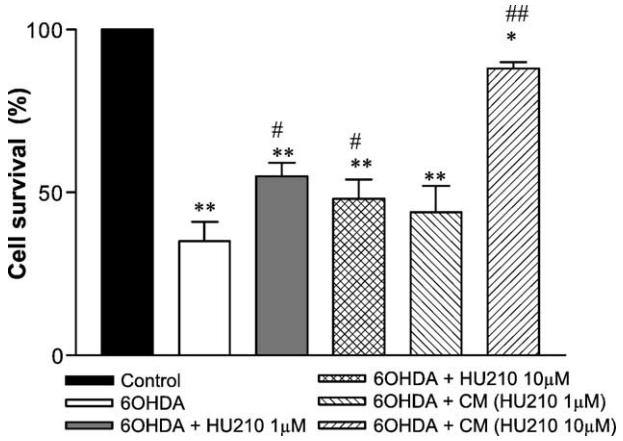

Fig. 2. Induction of cell death by 6-hydroxydopamine exposure of cultured mouse cerebellar granule neurons, and protective effects of HU-210 when added directly to neuronal cultures or through the generation of conditioned media (CM) from mixed glial cell cultures. Values are means ± SEM of 4 to 6 independent experiments each carried out in triplicate. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test (*$P < 0.05$, **$P < 0.005$ versus controls; #$P < 0.05$, ##$P < 0.005$ versus 6-hydroxydopamine alone).

1999), studies that frequently, although not in all cases, revealed the occurrence of an overactivity of this system compatible with the hypokinesia characteristic of this disease (for a review, see Sethi, 2002). This overactivity, however, did not occur within 2 weeks after the lesion, as reported now. This period of time is significantly shorter than the periods used in previous studies reporting CB_1 receptor up-regulation (Lastres-Becker et al., 2001; Mailleux and Vanderhaeghen, 1993; Romero et al., 2000). We also found decreased mRNA levels for VR1 receptors 2 weeks after the lesion, a fact that was expected because of the location of this receptor subtype in nigrostriatal neurons (Mezey et al., 2000) that degenerate by 6-hydroxydopamine application. In our view, this shorter time period is more appropriate for the examination of the protective action of cannabinoids in this disease, since it mimics the first phases of PD in humans, probably the only one at which the neuroprotection by cannabinoids might be achieved. This lack of changes in CB_1 receptors at 2 weeks post-lesion indicates that the up-regulation only occurs when dopaminergic injury is strong and when there are less possibilities for a protectant therapy.

Previous studies have addressed the hypothetical efficacy of cannabinoid agonists or antagonists by reducing motor symptoms in PD (Di Marzo et al., 2000; Gilgun-Sherki et al., 2003; Maneuf et al., 1997; Meschler et al., 2001; Sañudo-Peña et al., 1998) or by alleviating the dyskinesia that develops after chronic dopaminergic replacement therapy (Brotchie, 1998, 2000; Ferrer et al., 2003; Fox et al., 2002; Sieradzan et al., 2001). However, no evidence exists, to our knowledge, of a potential usefulness of cannabinoids to delay/arrest the progress of neurodegeneration in this disease, despite their well-demonstrated neuroprotectant efficacy in other models of acute or chronic degeneration (see references in Introduction). Here, we present the first evidence that Δ^9-THC also acted as a neuroprotective substance in rats with hemiparkinsonism. Thus, the chronic administration of this cannabinoid to rats, starting 16 h (to avoid potential chemical interferences between the cannabinoid and the toxin) after they were subjected to unilateral lesions of the nigrostriatal dopaminergic neurons with 6-hydroxydopamine, produced a significant recovery in the impairment of dopaminergic transmission caused by the toxin, likely indicating a reduction of dopaminergic cell death. This recovery modified neurochemical levels that become now, in most cases, similar or close to those observed in the ipsilateral structures of sham-operated animals. As we did not observe any changes of these neurochemical parameters in contralateral structures (all intact) by cannabinoid treatment, we assume that the changes observed in the lesioned structures are indicative of neuroprotection rather than of the occurrence of up-regulatory effects in surviving neurons (if this were the case, the effects would be recorded in both ipsilateral and contralateral structures). Interestingly, this recovery seemed to be persistent and irreversible since the interruption of chronic Δ^9-THC treatment after 2 weeks did not result in a relapse of the dopaminergic injury. This last observation is also another data in support that the effect of cannabinoids in 6-hydroxydopamine-lesioned rats is produced by prevention of cell death and/or rescue of affected neurons, and does not indicate the occurrence of an upregulatory response of surviving neurons. If this were the case, the interruption of Δ^9-THC treatment should have resulted in a loss of these effects and, then, dopaminergic parameters should have diminished again. Also supporting the view that the effect of Δ^9-THC was produced by the arrest of cell death and/or the rescue of affected neurons is the fact that this cannabinoid has been shown already capable to increase the number of TH-containing neurons in studies with cultured fetal mesencephalic neurons (Hernández et al., 2000). On the other hand, it is less probable, but we cannot completely rule out, in absence of additional studies, that these data might also reflect an axonal sprouting response in surviving cell bodies, as has been previously reported that specific cannabinoids may produce in other pathological conditions (Zalish and Lavie, 2003).

As mentioned above, the present observation that chronic Δ^9-THC treatment reduced the magnitude of dopaminergic injury in rats with hemiparkinsonism, is concordant with previous data showing that plant-derived, synthetic, or endogenous cannabinoids were neuroprotectant in a variety of in vivo and in vitro models of neuronal injury. However, it has been demonstrated that the mechanisms involved in these effects might be diverse, from events not mediated by cannabinoid receptors (NMDA antagonism, antioxidant properties; see Grundy et al., 2001, and Mechoulam et al., 2002a,b for review) up to CB_1 receptor-mediated phenomena (inhibition of glutamate release, stimulation of GABA action, reduction of Ca^{++} influx, hypothermia, vascular effects, and others; see also Grundy et al., 2001, and Mechoulam et al., 2002a,b). The protective effects observed for Δ^9-THC in the present study might be the result of an action independent of CB_1 receptors. This can be concluded from the fact that the two plant-derived cannabinoids, Δ^9-THC and CBD, tested here were equally effective in attenuating the dopaminergic impairment following to the lesion with 6-hydroxydopamine, despite their differences in the affinity for CB_1 receptors (CBD has negligible activity at this receptor subtype; see Bisogno et al., 2001; Pertwee, 1997). A similar observation was made by Hampson et al. (1998) who examined the neuroprotective effects of Δ^9-THC and CBD in rat cortical neuron cultures exposed to toxic levels of glutamate. These authors found that the ability of both cannabinoids to provide neuroprotection is CB_1 receptor-independent and based on the antioxidant properties of both compounds which are relatively equivalent (Hampson et al., 1998) and comparable, or even superior, to those reported for classic antioxidants such as ascorbate or α-tocopherol (Hampson et al., 2000). Further studies by Chen and Buck (2000) and Marsicano

et al. (2002) also reported that cannabinoids protect cells from oxidative stress basically through a CB_1 receptor-independent mechanism. Therefore, our data, collectively, are concordant with the notion that these two plant-derived cannabinoids may function as neuroprotectant in PD based on their capability to reduce oxidative stress which represents a major hallmark in the pathogenesis of this disease (Blum et al., 2001). However, cannabinoids may also be effective in PD through mechanisms other than their antioxidant properties. For instance, the activation of non-CB_1/non-CB_2 receptors may be of importance and, in view of the potent anti-inflammatory action of both cannabinoids, in particular CBD (Malfait et al., 2000), the blocking of the production of various factors associated with inflammation (nitric oxide, TNFα, and others) by these cannabinoids may be also relevant (see below). Even, it would be conceivable that the protective effect exerted by CBD might be produced through its recently reported ability to block anandamide breakdown and its uptake thus elevating anandamide levels (Bisogno et al., 2001) or, even, by its modest affinity for the CB_2 receptor subtype (Pertwee, 1997). In this sense, we have preliminary evidence that the blockade of the endocannabinoid inactivation with UCM707, a selective inhibitor of the endocannabinoid transport system (López-Rodríguez et al., 2003) that does not possess any antioxidant properties, did not reduce dopaminergic impairment caused by the application of 6-hydroxydopamine (data not shown). This discards that CBD might also act through blocking the endocannabinoid inactivation. As regards to a potential involvement of CB_2 receptors, it is important to remark that recent data have demonstrated that this receptor subtype, although relatively absent of the brain parenchyma in healthy conditions, is markedly expressed as a consequence of reactive astrocytosis and/or microglial cell activation that are produced by a degenerative insult (Benito et al., 2003). Other data have related CB_2 receptor to events involved in the progression or arrest of neurodegeneration, for instance, by influencing microglial cell migration at neuroinflammatory lesion sites (Walter et al., 2003). Therefore, further studies will have to explore whether other types of cannabinoids might provide neuroprotection by mechanisms distinct of those initially offered by Δ^9-THC or CBD, and, in particular to examine the role of the CB_2 receptor subtype. The data obtained in the second group of experiments of this study support this possibility. These experiments were aimed at exploring whether the protective effects of cannabinoids against the in vivo toxicity of 6-hydroxydopamine might be also observed in vitro and exerted by regulating glial trophic support to neurons (i.e., by increasing prosurvival factors, and/or by reducing cytotoxic ones). Our results strongly support both hypotheses. First, HU-210 was able to reduce 6-hydroxydopamine induced cell death when added directly to cultured cerebellar neurons although these effects were small. We have recently described the same neuroprotective effect exerted by HU-210 in cultured cortical neurons subjected to excitotoxic stimulus and found that this effect is mediated by phosphatidylinositol 3-kinase/Akt signaling pathway (Molina-Holgado et al., in press). The interest of this last observation is that this signaling pathway has been strongly implicated in survival signaling in many cell types including neurons and glial cells (Brunet et al., 2001). Second, we have also found that glial cells are important in mediating part of the neuroprotective effects of cannabinoids against the in vitro toxicity of 6-hydroxydopamine. This can be concluded from the fact that conditioned media, generated by exposure of mixed glial cells to HU-210, produced a greater reduction of the rate of neuronal cell death induced by 6-hydroxydopamine when they were added to neuronal cultures than in the case of direct exposure of these neuronal cultures to HU-210. In addition, in this last case, the effect of HU-210 was not dose-dependent thus indicating possible overlapping of different mechanisms activated by this cannabinoid. By contrast, there was a clear dose-dependent response when the cannabinoid was administered to mixed glial cell cultures, possibly indicating that it could be receptor-mediated, either CB_1 or CB_2 because of the lack of selectivity of HU-210 and because of the presence of both cannabinoid receptor subtypes in glial cells. It is well known that conditioned media generated by cultured glial cells are per se able to protect neurons from spontaneous and toxin-induced cell death (De Bernardo et al., 2003). This is likely related to the presence of prosurvival mediators (i.e., anti-inflammatory molecules) or the lack of death-induced factors (i.e., nitric oxide, TNF-α, pro-inflammatory cytokines). It is possible that, in our study, the activation of CB_1 and/or CB_2 receptors by HU-210 in mixed glial cell cultures dose-dependently increased the presence of these prosurvival mediators and/or reduced that of death-induced factors, thus producing a greater neuronal survival. In support of this idea, it has been reported that cannabinoids inhibit the production of nitric oxide and pro-inflammatory cytokines (for a review, see Guzmán et al., 2001; Smith et al., 2000; Waksman et al., 1999). For instance, we have recently demonstrated that interleukin-1 receptor antagonist, an important anti-inflammatory cytokine that protects against experimentally-induced ischemic, excitotoxic, and traumatic brain insults, is produced in response to cannabinoid receptor activation in primary cultured glial cells (Molina-Holgado et al., 2003). Interestingly, cannabinoid receptor activation failed to do this in knockout mice for this anti-inflammatory cytokine (Molina-Holgado et al., 2003). In the same line of reasoning, we have also observed that 6-hydroxydopamine is also able to produce neuronal death through glial cell-mediated effects since neuronal cultures incubated with conditioned media obtained after adding this toxin to cultured mixed glial cells, showed similar rates of cell death than when the toxin was directly added to neuronal cultures. It is possible that interleukin-1β might be one of these critical factors since the above neurotoxic effects of 6-hydroxydopamine were significantly reduced when cultures were obtained from interleukin-1β-deficient mice (unpublished results). On the other hand, some studies reported that cannabinoids are also protective in glial cells and that this effect is mediated by activation of phosphatidylinositol 3-kinase/Akt signaling pathway (Gomez del Pulgar et al., 2002). As mentioned above, we have recently demonstrated that this mechanism, which has been strongly related to survival signaling (Brunet et al., 2001), is also mediating the protective effects of cannabinoids in neurons (Molina-Holgado et al., in press). It is possible that the greater neuroprotective effects observed for HU-210 when used to generate conditioned media than when added directly to neuronal cultures may be indicative of a more efficient activation of that signaling pathway by cannabinoids in glial cells than in neurons.

In summary, our results are compatible with a potential neuroprotective action of Δ^9-THC against the progressive degeneration of nigrostriatal dopaminergic neurons occurring in PD, a neurodegenerative disorder with a useful symptomatic therapy but, as other neurodegenerative diseases, lacking an efficient neuroprotectant therapy. However, the fact that the same neuroprotective effects were elicited by CBD, a plant-derived cannabinoid with negligible affinity for the cannabinoid receptors, suggests a major involvement of CB_1 receptor-independent mechanisms, possibly

based on the antioxidative properties of both compounds and/or the effects associated with their well known anti-inflammatory activity, such as lowering the production of TNFα, nitric oxide, and other biologically active molecules. It is important to remark that the fact that CBD was equivalent to Δ^9-THC in reducing dopaminergic injury in PD supports the assumption that CBD would be more advantageous for a potential neuroprotectant therapy in this disease, since it can be used at higher doses and for longer times than those possible with Δ^9-THC, due to its lack of psychoactivity. An additional advantage for CBD is that its use in prolonged treatments does not induce tolerance (Malfait et al., 2000), a phenomenon often observed with Δ^9-THC (Adams and Martin, 1996). In addition, the evidence provided by in vitro studies also indicates the occurrence of additional mechanisms of neuroprotection by cannabinoids that would include a modulation of glial function that would be effective in reducing inflammatory responses that usually accompany neurodegenerative insults.

Acknowledgments

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