Research Report

Evaluation of the neuroprotective effect of cannabinoids in a rat model of Parkinson's disease: Importance of antioxidant and cannabinoid receptor-independent properties

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ABSTRACT

We have recently demonstrated that two plant-derived cannabinoids, Δ^9-tetrahydrocannabinol and cannabidiol (CBD), are neuroprotective in an animal model of Parkinson's disease (PD), presumably because of their antioxidant properties. To further explore this issue, we examined the neuroprotective effects of a series of cannabinoid-based compounds, with more selectivity for different elements of the cannabinoid signalling system, in rats with unilateral lesions of nigrostriatal dopaminergic neurons caused by local application of 6-hydroxydopamine. We used the CB_1 receptor agonist arachidonyl-2-chloroethylamide (ACEA), the CB_2 receptor agonist HU-308, the non-selective agonist WIN55,212-2, and the inhibitors of the endocannabinoid inactivation AM404 and UCM707, all of them administered i.p. Daily administration of ACEA or WIN55,212-2 did not reverse 6-hydroxydopamine-induced dopamine (DA) depletion in the lesioned side, whereas HU-308 produced a small recovery that supports a possible involvement of CB_2 but not CB_1 receptors. AM404 produced a marked recovery of 6-hydroxydopamine-induced DA depletion and tyrosine hydroxylase deficit in the lesioned side. Possibly, this is caused by the antioxidant properties of AM404, which are derived from the presence of a phenolic group in its structure, rather than by the capability of AM404 to block the endocannabinoid transporter, because UCM707, another transporter inhibitor devoid of antioxidant properties, did not produce the same effect. None of these effects were observed in non-lesioned contralateral structures. We also examined the timing for the effect of CBD to provide neuroprotection in this rat model of PD. We found that CBD, as expected, was able to recover 6-hydroxydopamine-induced DA depletion when it was administered immediately after the lesion, but it failed to do that when the treatment started 1 week later. In addition, the effect of CBD implied an upregulation of mRNA levels for Cu,Zn-superoxide dismutase, a key enzyme in endogenous defenses against oxidative stress. In summary, our results indicate that those cannabinoids having antioxidant cannabinoid receptor-independent properties provide neuroprotection against the progressive degeneration of nigrostriatal dopaminergic neurons occurring in PD. In addition, the activation of CB_2 (but not CB_1)

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receptors, or other additional mechanisms, might also contribute to some extent to the potential of cannabinoids in this disease.

1. Introduction

We recently demonstrated that certain cannabinoids may be neuroprotectant in Parkinson's disease (PD) (Lastres-Becker et al., 2005), 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). Using a rat model of this disease generated by unilateral injections of 6-hydroxydopamine into the medial forebrain bundle (see Schober, 2004, for review), we observed that two plant-derived cannabinoids, Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD), were able to attenuate the dopamine (DA) depletion and tyrosine hydroxylase (TH) deficits caused by the toxin in the striatum that are indicative of the degree of neurodegeneration of nigrostriatal dopaminergic projections (Lastres-Becker et al., 2005). A priori the neuroprotection provided by both cannabinoids in this disease might be due to the well-described antiglutamatergic (mainly mediated by CB_1 receptors), anti-inflammatory (which preferentially involve CB_2 receptors) and/or antioxidant (likely cannabinoid receptor-independent) properties of cannabinoids that have been demonstrated to operate against acute or chronic neurodegeneration (see Fernández-Ruiz et al., 2005, 2007, for review). In this sense, the fact that the neuroprotective effect of cannabinoids found in vivo in hemiparkinsonian rats was observed for Δ^9-THC (Lastres-Becker et al., 2005), which is able to bind both CB_1 and CB_2 receptors, but also for CBD (Lastres-Becker et al., 2005), which does not bind either cannabinoid receptor subtype (Pertwee, 1997; Bisogno et al., 2001; Mechoulam and Hanus, 2002), suggested that it would be likely originated by the antioxidant and cannabinoid receptor-independent properties of both plant-derived cannabinoids (Hampson et al., 1998, 2000; Marsicano et al., 2002). However, in our precedent study (Lastres-Becker et al., 2005), we also explored the potential of cannabinoids against the in vitro neurotoxicity of 6-hydroxydopamine and we found that HU-210, a non-selective cannabinoid receptor agonist, was also able to increase neuronal survival. HU-210 also has antioxidant properties (Marsicano et al., 2002), but, in our study (Lastres-Becker et al., 2005), it acted through another mechanism by increasing the trophic support exerted by glial cells on neurons, an effect that would be presumably CB_1 or CB_2 receptor-mediated. This suggests the involvement of additional mechanisms for cannabinoids to provide neuroprotection in PD (Lastres-Becker et al., 2005).

Based on this previous evidence, the present study was designed to examine the ability to provide neuroprotection in vivo in PD of a series of cannabinoid-based compounds with more selectivity for different elements of the cannabinoid signalling system. We used the CB_1 receptor agonist arachidonyl-2-chloroethylamide (ACEA; Hillard et al., 1999), the CB_2 receptor agonist HU-308 (Hanus et al., 1999), the non-selective agonist WIN55,212-2 (Pertwee, 1997), and the inhibitors of the endocannabinoid inactivation AM404 (Beltramo et al., 1997) and UCM707 (López-Rodríguez et al., 2003). We used a rat model of PD that replicates the progressive death of nigrostriatal dopaminergic neurons characteristic of PD patients (for review, see Blum et al., 2001). This model consisted of unilateral injections into the medial forebrain bundle of 6-hydroxydopamine, a toxin that produces a progressive death of these neurons and replicates the characteristic motor abnormalities and molecular dysfunctions of this disease (for review, see Blum et al., 2001; Schober, 2004). The advantage of this model for studies of neuroprotection is that the contralateral structures may be used as an internal control to test the effects of substances in the absence of lesion, allowing to differentiate between neuroprotective effects (those found only in the lesioned side) and upregulatory responses (those also found in the non-lesioned side) (see details in Lastres-Becker et al., 2005). In a first experiment, ACEA, HU-308, WIN55,212-2, AM404 or UCM707 were 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. In a second experiment, we evaluated the timing for the neuroprotective effect of antioxidant cannabinoids such as CBD, a compound that, as mentioned above, exhibited a potent neuroprotective effect in this rat model (Lastres-Becker et al., 2005). In this experiment, we examined the differences between the effect of this cannabinoid when administered immediately after the lesion, as in our previously published study (Lastres-Becker et al., 2005), or starting the treatment at 1 week post-lesion. We also examined whether the neuroprotective effect of CBD was produced by increasing the endogenous defenses against oxidative stress, by examining the expression of Cu,Zn-superoxide dismutase (Cu,Zn-SOD), a key enzyme in this response, that accounts for a 90% of the total SOD (Noor et al., 2002). The rationale for this last analysis is based on the great physiological significance and therapeutic potential assigned to this enzyme and, in particular, on the data that indicate the existence of alterations in Cu,Zn-SOD function in several neurodegenerative diseases, including PD (see Noor et al., 2002, for review). This makes this enzyme a good candidate to explore potential substrates involved in the antioxidant and neuroprotective effects of CBD or other cannabinoids. In all these experiments, 6-hydroxydopamine-induced death of nigrostriatal dopaminergic neurons was evaluated by analyzing the magnitude of DA depletion in the caudate–putamen and, in some cases, also by analyzing mRNA levels (in the substantia nigra) and activity (caudate–putamen) of TH, the rate-limiting enzyme for DA synthesis in these neurons.

2. Results

Unilateral injections of 6-hydroxydopamine into the medial forebrain bundle produced, at 2 weeks post-injection, a

significant depletion of DA contents and a reduction of TH activity in the striatum of the lesioned side, which was accompanied by a reduction in TH-mRNA levels in the substantia nigra. As expected, the effects of 6-hydroxydopamine were asymmetrical since none of these deficits occurred in the contralateral structures (data not shown). Values ranged from 40% to 60% in the lesioned side over the equivalent values in the non-lesioned side (data not shown). In all cases, the parameter obtained from calculating the percentage of each individual value in the lesioned side over the corresponding value in the contralateral non-lesioned side may be used as index of the degree of DA injury following 6-hydroxydopamine application. Using this parameter, we are able to easily differentiate, when potential therapeutic agents are tested in this model, whether the effects are neuroprotective (found only in the lesioned side) or whether the compounds produce upregulatory responses (also found in the non-lesioned side). We then found that the administration of ACEA (a selective CB_1 receptor agonist) or WIN55,212-2 (a non-selective cannabinoid receptor agonist) during 2 weeks, starting 16 h after the application of 6-hydroxydopamine, did not reverse the DA depletion caused by the toxin in the lesioned striatum (Fig. 1). By contrast, HU-308 (a selective CB_2 receptor agonist) produced a small recovery in DA depletion ($F_{(2,18)} = 3.793$, $p < 0.05$; see Fig. 1) that would support a possible involvement of CB_2 but not CB_1 receptors in the neuroprotective effects of cannabinoids in this model. However, this would be only a modest effect since it was not evident for other parameters that also indicate the degree of DA injury, such as TH activity in the caudate–putamen and TH-mRNA levels in the substantia nigra (Fig. 2). This was not the case of AM404, an inhibitor of the endocannabinoid transporter, since
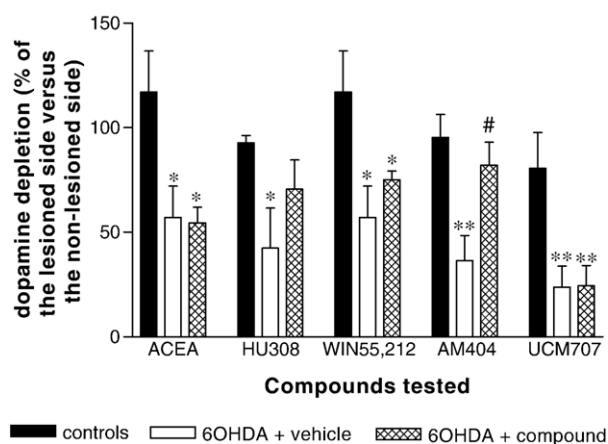

Fig. 1 – Effects of 2 weeks of daily administration of ACEA (1 mg/kg), HU-308 (5 mg/kg), WIN55,212-2 (3 mg/kg), AM404 (5 mg/kg), UCM707 (5 mg/kg), or their corresponding vehicle, on dopamine contents in the caudate–putamen of rats with unilateral lesions of the nigrostriatal dopaminergic neurons caused by local injection of 6-hydroxydopamine. Details are in the text. Values are expressed as means ± SEM of 6–8 animals per group. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test (*$p < 0.05$, **$p < 0.01$ vs. control rats; #$p < 0.05$ vs. vehicle-injected 6-hydroxydopamine-lesioned rats).
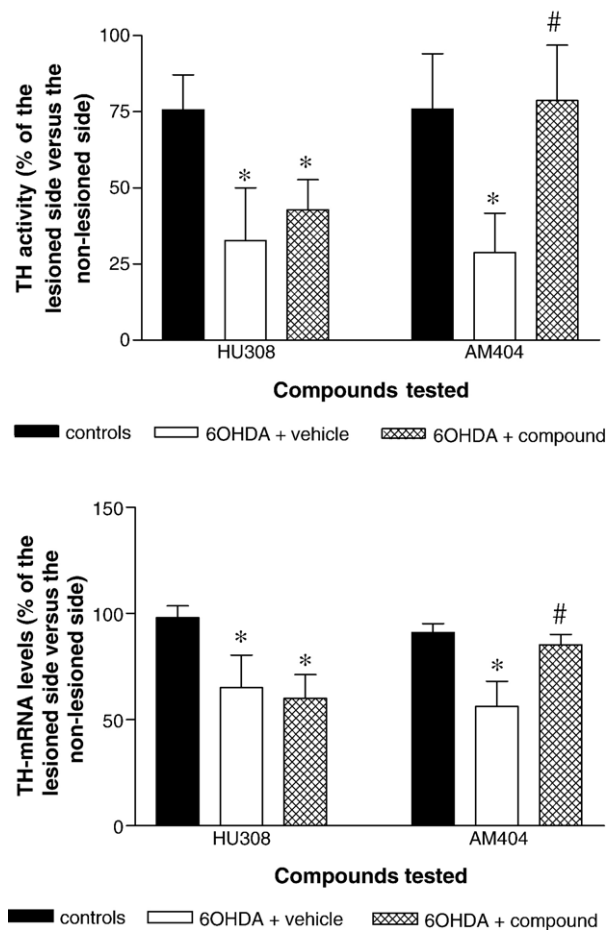

Fig. 2 – Effects of 2 weeks of daily administration of HU-308 (5 mg/kg), AM404 (3 mg/kg), or their corresponding vehicle on TH activity in the caudate–putamen and TH-mRNA levels in the substantia nigra of rats with unilateral lesions of the nigrostriatal dopaminergic neurons caused by local injection of 6-hydroxydopamine. Details are in the text. Values are expressed as means ± SEM of at least 6–8 animals per group. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test (*$p < 0.05$ vs. control rats; #$p < 0.05$ vs. vehicle-injected 6-hydroxydopamine-lesioned rats).

this compound produced a marked recovery of 6-hydroxydopamine-induced DA depletion ($F_{(2,17)} = 7.373$, $p < 0.01$; see Fig. 1) and TH deficits (TH-mRNA levels: $F_{(2,19)} = 3.894$, $p < 0.05$; TH activity: $F_{(2,17)} = 3.755$, $p < 0.05$; see Fig. 2) in the lesioned side. However, the fact that another endocannabinoid transporter inhibitor, UCM707, did not produce the same effect (Fig. 1), suggest that AM404 effects would be likely caused more by its antioxidant properties derived from the presence of a phenolic group in its structure rather than by its capability to block the endocannabinoid transporter. UCM707, which contains a heterocycle bound to arachidonic acid, is devoid of antioxidant properties. On the other hand, it is important to remark again that the effects found for AM404 and, to a lesser extent, for HU-308 were evident when we compared the percentage in the lesioned side over the non-lesioned side for each parameter (DA depletion or TH deficits). However, it must be

stressed that the changes found for both the effects of 6-hydroxydopamine and the recovery caused by these two cannabinoids was always the consequence of changes recorded in the lesioned side (data not shown). In all cases, none of these effects were observed in non-lesioned contralateral structures, thus discarding a potential upregulatory effect.

Finally, we also examined the timing for the effect of CBD. This cannabinoid was used here as an example of those cannabinoids that provided neuroprotection in this rat model of PD due to its antioxidant properties. We found that CBD was
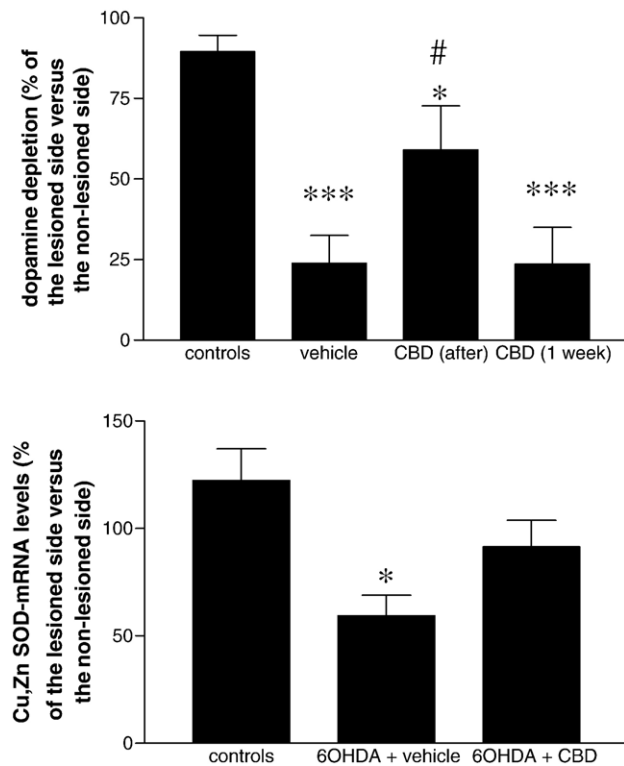

Fig. 3 – Top panel: Effects of daily administration of CBD (3 mg/kg) or vehicle, starting immediately after (16 h later) or 1 week after the lesion, on dopamine contents in the caudate–putamen of rats with unilateral lesions of the nigrostriatal dopaminergic neurons caused by local injection of 6-hydroxydopamine. Details are in the text. Values in controls and in 6-hydroxydopamine-lesioned rats treated with vehicle come from two separate experiments (one starting CBD treatment immediately after lesion and another starting 1 week later). As these two groups showed equivalent values in both experiments, they were combined for statistical analysis and presentation. Bottom panel: Effects of 2 weeks of daily administration of CBD (3 mg/kg) or vehicle on mRNA levels for Cu,Zn-SOD in the substantia nigra of rats with unilateral lesions of the nigrostriatal dopaminergic neurons caused by local injection of 6-hydroxydopamine. Details are in the text. Values are expressed as means ± SEM of at least seven animals per group. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test ($*p < 0.05$, $**p < 0.01$, $***p < 0.005$ vs. control rats; $#p < 0.05$ vs. vehicle-injected 6-hydroxydopamine-lesioned rats).

able to recover 6-hydroxydopamine-induced DA depletion when it was administered immediately after the lesion ($F(3,42) = 16.10$, $p < 0.0001$; see Fig. 3). However, CBD failed to do that when the treatment started 1 week after the injection of 6-hydroxydopamine (Fig. 3). We also explored whether the neuroprotective effect of CBD, when administered immediately after the lesion, was related to changes in the status of endogenous defenses against oxidative stress. We found that, as expected, the application of 6-hydroxydopamine significantly reduced mRNA levels for Cu,Zn-SOD in the substantia nigra (Fig. 3), a key enzyme in endogenous defenses against oxidative stress and a parameter very representative of the degree of neuronal injury caused by oxidative stress in PD. The administration of CBD originated an upregulation of mRNA levels for Cu,Zn-SOD in the substantia nigra ($F(2,21) = 4.697$, $p < 0.05$; see Fig. 3). Again, it must be stressed that the effects caused by the application of 6-hydroxydopamine and also by the administration of CBD were always evident in the ipsilateral lesioned side but they did not occur in the contralateral non-lesioned side.

3. Discussion

Parkinson's disease is an adult-onset neurodegenerative disorder characterized by a preferential loss of the dopaminergic neurons of the *substantia nigra pars compacta* that leads to a severe dopaminergic denervation of the striatum (for review, see Sethi, 2002). Although the etiology of PD is presently unknown, major pathogenic processes, which trigger the progressive loss of nigral dopaminergic neurons, are oxidative stress, mitochondrial dysfunction and inflammatory stimuli (Sherer et al., 2001; McGeer et al., 2001). The present therapeutics in this disease are essentially addressed to ameliorate motor symptoms (mainly, dopaminergic replacement therapy) and are not able to block the degenerative process, although classic strategies used in acute or chronic neurodegeneration, such as antioxidants, NMDA receptor antagonists, calcium channel blockers or antiinflammatory molecules, have been also employed in PD (for review, see Vajda, 2002).

We recently presented the first evidence for an *in vivo* neuroprotective action of cannabinoids in an animal model of this disease, rats with hemiparkinsonism caused by the unilateral application of 6-hydroxydopamine (Lastres-Becker et al., 2005). Two cannabinoids were examined in this previous study, Δ^9-THC and CBD, and both produced a significant recovery in the impairment of dopaminergic transmission typical of hemiparkinsonian rats. We have also provided sufficient evidence to demonstrate that this cannabinoid-induced recovery in dopaminergic transmission is produced by a reduction of dopaminergic cell death and not by inducing upregulatory responses in surviving neurons (Lastres-Becker et al., 2005). In addition, the fact that both Δ^9-THC and CBD were equally effective in attenuating the dopaminergic impairment following to the lesion with 6-hydroxydopamine, despite their differences in the affinity for the CB$_1$ and CB$_2$ receptors (CBD has negligible activity at cannabinoid receptors; see Pertwee, 1997; Bisogno et al., 2001; Mechoulam and Hanus, 2002), would speak in favour of an involvement of the antioxidant and cannabinoid receptor-independent

properties of certain cannabinoids (see details in the Introduction). This would agree with the data reported 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.

The assumption of this idea would *a priori* exclude the involvement of other cannabinoid receptor-mediated mechanisms (inhibition of glutamate release, reduction of Ca^{2+} influx, hypothermia, antiinflammatory actions, vascular effects, and others; for review, see Grundy, 2002; Mechoulam et al., 2002; Fernández-Ruiz et al., 2005) in the neuroprotection provided by these two plant-derived cannabinoids in hemiparkinsonian rats (Lastres-Becker et al., 2005). However, some additional observations would support that other cannabinoids that do not exhibit these antioxidant properties might be also effective in PD through additional mechanism(s) (see details in the Introduction). This possibility has been examined in the present study using a strategy based on the analysis of neuroprotective potential of compounds having more selectivity for different elements of the cannabinoid signalling system (see a summary in Table 1). This has allowed to conclude: (i) that CB_1 receptors do not appear to provide neuroprotection in hemiparkinsonian rats, since ACEA, a selective agonist, or WIN55,212-2, a non-selective agonist, both devoid of antioxidant properties, were not effective in this animal model; (ii) that CB_2 receptors might provide some degree of neuroprotection, as revealed from the data obtained with HU-308, a selective agonist of this receptor subtype (Hanus et al., 1999), although their contribution is relatively more modest than the effect produced by antioxidant cannabinoids; (iii) that inhibition of the endocannabinoid inactivation process, which would allow to elevate and prolong the endocannabinoid action (de Lago et al., 2002; López-Rodríguez et al., 2003), did not provide neuroprotection, as revealed the lack of effects of a selective transporter inhibitor UCM707 (see de Lago et al., 2006, for more details); and (iv) that AM404, despite its capability to block the transporter, would provide neuroprotection in hemiparkinsonian rats mainly due to its reported antioxidant properties derived from the presence of a phenolic group in its structure (Marsicano et al., 2002). The five compounds tested here have pharmacokinetic properties very similar to those of classic cannabinoids (Pertwee, 1997), so it is unlikely that the lack of effects of some of them may be due to the fact that they did not reach effective concentrations in the brain after i.p. administration. Therefore, the present data are essentially a demonstration that antioxidant and receptor-independent properties of certain cannabinoids are an effective mechanism to reduce neuronal death in PD, a disorder where oxidative stress represents an extremely important event in the pathogenesis (see Przedborski, 2005, for review). However, this finding deserves several additional comments.

Table 1 – Summary of neuroprotective effects of cannabinoid-based compounds in hemiparkinsonian rats		
Compound	Major characteristics	Effect against *in vivo* 6-hydroxydopamine toxicity
ACEA	CB_1 receptor agonist [a]	No
HU-308	CB_2 receptor agonist [b]	Yes (modest effects)
WIN55,212-2	CB_1/CB_2 receptor agonist [c]	No
AM404	Inhibitor of the endocannabinoid transporter [d] TRPV1 receptor agonist [e] Antioxidant [f]	Yes (antioxidant, but also COX2- or TRPV1 receptor-mediated)
UCM707	Inhibitor of the endocannabinoid transporter [g]	No
CBD	Poor agonist at cannabinoid receptors [h] Antioxidant [f,h]	Yes [i] (antioxidant, but also COX2- or TRPV1 receptor-mediated) Effective immediately after lesion Increases expression of Cu,Zn-SOD
Δ^9-THC [i]	CB_1/CB_2 receptor agonist [c] Antioxidant [f]	Yes [i] (antioxidant or COX2-mediated)

[a] Hillard et al. (1999).
[b] Hanus et al. (1999).
[c] Pertwee (1997).
[d] Beltramo et al. (1997).
[e] Zygmunt et al. (2000).
[f] Marsicano et al. (2002).
[g] López-Rodríguez et al. (2003).
[h] Mechoulam and Hanus (2002).
[i] Lastres-Becker et al. (2005).

First, the antioxidant and neuroprotective action shown by Δ^9-THC (Lastres-Becker et al., 2005), CBD (Lastres-Becker et al., 2005, and also in present data) and AM404 (present data) in hemiparkinsonian rats would be a cannabinoid receptor-independent phenomenon that would be presumably related to a potential of these compounds as scavengers of free radicals due to their structural characteristics (Hampson et al., 2000). However, in the present study, we also wanted to explore the status of endogenous defenses against oxidative stress in hemiparkinsonian rats and how these defenses are influenced by the use of antioxidant cannabinoids that act neuroprotectively in this animal model. We selected Cu,Zn-SOD, an enzyme that plays a key role in neuronal protection against the damaging effects of superoxide anions (Noor et al., 2002, for review). Our starting hypothesis was that CBD (or other antioxidant cannabinoids), if it acts by reducing oxidative stress, it might also facilitate Cu,Zn-SOD function. We found that the application of 6-hydroxydopamine reduced mRNA levels of Cu,Zn-SOD in the substantia nigra, to an extent similar to that previously reported by Kunikowska and Jenner (2001). This is concordant with the notion that endogenous defenses against oxidative stress, including Cu,Zn-SOD, decrease in PD patients (see Noor et al., 2002, for review). The administration of CBD reversed this decrease. A

priori one may interpret this observation as an indication of the neuroprotective effect of CBD since the increase of mRNA levels for Cu,Zn-SOD is proportional to the number of surviving neurons. The fact that the effect of CBD was observed in the ipsilateral but not in the contralateral side supports this option. However, other interpretations are also possible, although they will need additional research. For instance, it is also possible that CBD, by reducing the levels of free radicals as a result of its scavenger function, might indirectly improve Cu,Zn-SOD function recovering the deficit caused in this enzyme by the toxin in animals and, eventually, by the disease in patients (Noor et al., 2002, for review). However, it is also possible that CBD might activate several intracellular signals that enhance the synthesis of Cu,Zn-SOD. In that case, this enzyme would be the major responsible for the reduction of superoxide radicals thus originating the neuroprotective effect. The induction of Cu,Zn-SOD by CBD would be equivalent to that reported for the DA agonist pergolide (Ihara et al., 1999), which was able to act as a free radical scavenger (Nishibayashi et al., 1996) by inducing the expression of this key antioxidant enzyme (Clow et al., 1992). In the same line of reasoning, Patel et al. (2002) reported that overexpression of Cu,Zn-SOD was associated with a decrease of cell death induced by several apoptotic stressers (hypoxia–reperfusion, staurosporine or γ-interferon).

On the other hand, although the neuroprotective effects exerted in hemiparkinsonian rats by Δ^9-THC (Lastres-Becker et al., 2005), CBD (Lastres-Becker et al., 2005, and also present data) and AM404 (present data) seem to critically depend on the antioxidant properties of these compounds, there is room for another alternative mechanisms that might explain the action of these cannabinoids (see a summary in Table 1). One of them, the possibility that TRPV1 receptors might be involved, is supported by three facts: (i) that these receptors have been linked to neuroprotection in different conditions (Veldhuis et al., 2003; Pegorini et al., 2005, 2006); (ii) that they can be activated by AM404 (Zygmunt et al., 2000; De Petrocellis et al., 2000; Ross et al., 2001) or CBD (Bisogno et al., 2001); and (iii) that TRPV1 receptors have been identified in nigrostriatal dopaminergic neurons (Mezey et al., 2000) and are affected by the lesion of these neurons (Lastres-Becker et al., 2005). However, the involvement of TRPV1 receptors would be circumscribed only to the cases of AM404 or CBD since Δ^9-THC, the other cannabinoid that provided neuroprotection in this rat model of PD, is unable to bind and activate these receptors. Another alternative is the involvement of cyclooxygenase-2 (COX-2) inhibition. This is also based on a series of observations: (i) that induction of COX-2 have been reported to be instrumental in the neurodegeneration associated with PD (Teismann et al., 2003; Okuno et al., 2005); (ii) that AM404 is also an inhibitor of COX-2 (Hogestatt et al., 2005); and (iii) that we have preliminary and still unpublished evidence that CBD and Δ^9-THC may also inhibit COX-2 activity (Gallily and Mechoulam, unpublished data). Therefore, COX-2 inhibition might be a mechanism, other than directly acting as scavenger of free radicals, that might explain the potential of Δ^9-THC, CBD and AM404 as neuroprotective agents in PD. This hypothesis will be one of the major challenges for future experiments.

A second aspect deserving some comments is the potential role of CB$_2$ receptors in PD. The observation that HU-308 may also reduce the degree of dopamine depletion in the lesioned side, despite this effect was more modest than the effect produced by other compounds here or in our previous study (Lastres-Becker et al., 2005), would support a potential secondary involvement of CB$_2$ receptors. In this sense, it is important to remark that CBD may also have certain affinity at CB$_2$ receptors (Pertwee, 1997), so, if this receptor subtype is involved, part of the neuroprotection provided by CBD might be mediated by CB$_2$ receptors, but this is an option that would require further investigation. Part of this investigation would deal with the demonstration that CB$_2$ receptors, which are relatively absent of the brain parenchyma in healthy conditions, might be induced at the lesioned sites. This has been already demonstrated for other diseases, such as Alzheimer's disease or Huntington's disease (see Fernández-Ruiz et al., 2005, 2007, for details), where this receptor subtype is strongly induced/upregulated as a consequence of reactive astrocytosis and/or microglial cell activation that are produced by a degenerative insult (Benito et al., 2003; Fernández-Ruiz et al., 2007). The presence of CB$_2$ or CB$_2$-like receptors in the brain is becoming really a hot issue, even recent evidence suggests the presence of these receptors in non-pathological conditions. For example, CB$_2$ receptors have been identified in neural and oligodendroglial progenitors, in mature glial cells (microglia and astrocytes), and even in mature neuronal elements in the brain of several mammals, such as rodents, monkeys and humans (see Fernández-Ruiz et al., 2007, for a recent review). In neurodegenerative conditions, several data have related CB$_2$ receptor to events involved in the progression or arrest of brain damage, for instance, by influencing microglial cell migration at neuroinflammatory lesion sites (Walter et al., 2003). If CB$_2$ receptors are induced/upregulated in the substantia nigra as a consequence of the lesion caused by the injection of 6-hydroxydopamine, then, we would need to identify the cellular location of these receptors (i.e. microglia or astrocytes) and whether they might provide neuroprotection in hemiparkinsonian rats by reducing inflammatory events that usually accompany neurodegenerative insults. In support of this hypothesis, we have published some data demonstrating that a non-selective and highly potent agonist, HU-210, was able to protect neurons against the toxicity caused by 6-hydroxydopamine *in vitro*, an effect that was mainly produced through increasing the trophic support exerted by glial cells on neurons (Lastres-Becker et al., 2005).

Finally, it must be also remarked that the timing for cannabinoids to provide neuroprotection in hemiparkinsonian rats is restricted to specific periods during the course of neurodegeneration of the substantia nigra in these rats. If in our previous study (Lastres-Becker et al., 2005), we concluded that the cannabinoid effect was persistent and irreversible since the interruption of the treatment after 2 weeks did not result in a relapse of the dopaminergic injury, we may add now that the efficacy of cannabinoid effect depends on the initiation of the treatment immediately after the lesion. Thus, we found that CBD, when it was given to hemiparkinsonian rats 1 week after the lesion, was unable to recover the dopaminergic injury caused by 6-hydroxydopamine. This contrasts with the data obtained previously (Lastres-Becker et al., 2005), and corroborated again here, which demonstrated

that CBD was neuroprotective when the treatment was initiated at 16 h after the lesion. This observation implies that the protective effects of CBD, and possibly of the other cannabinoids, might be relevant only at very early steps during the progression of PD and when the lesion is still moderate, but they would not be relevant in advanced steps of this disease in humans. Therefore, CBD might be a preventive treatment for those patients with risk to develop PD, for instance patients bearing mutations in the different PARK genes or subjected to overexposure to several PD-related environmental toxins.

In summary, our results indicate that those cannabinoids having antioxidant cannabinoid receptor-independent properties provide neuroprotection against the progressive degeneration of nigrostriatal dopaminergic neurons occurring in PD. In addition, the activation of CB_2 (but not CB_1) receptors or other additional mechanisms (inhibition of COX-2 or activation of TRPV1 receptors) might also contribute to some extent to the potential of cannabinoids in this disease.

4.   Experimental procedures

4.1.   Animals, surgical procedures, treatments and sampling

4.1.1.   Animals
Male Sprague-Dawley rats (>8 weeks; approx. 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).

4.1.2.   Unilateral injection of 6-hydroxydopamine
After pretreatment (30 min before) with desipramine (25 mg/kg, i.p.), and under anaesthesia caused by i.p. administration of ketamine (60 mg/kg) and xylazine (4 mg/kg), rats were injected stereotaxically (coordinates: −2.5 mm anterior, −1.8 mm lateral from the bregma, −8.9 mm ventral from the dura mater; Paxinos and Watson atlas, 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), or were sham-operated. The injection was unilateral, the contralateral structures also serving as controls for the different analyses.

4.1.3.   Treatment with different cannabinoid-based compounds
CBD was purified from hashish in the Hebrew University laboratory as previously described (Gaoni and Mechoulam, 1971), HU-308 was kindly provided by Pharmos Ltd. (Rehovot, Israel), ACEA and AM404 were purchased from Tocris (Biogen Científica S.L., Madrid, Spain), WIN55,212-2 was purchased from Sigma Chem. (Madrid, Spain), and UCM707 was synthesized as previously described (López-Rodríguez et al., 2003). They were prepared in Tween 80–saline solution (1:16) for i.p. administration and used, in separate experiments, at specific doses according to their pharmacological potencies in equivalent injury models (see Grundy, 2002; Mechoulam et al., 2002; Fernández-Ruiz et al., 2005). The doses were 1 mg/kg weight

for ACEA, 3 mg/kg for CBD and WIN55,212-2, or 5 mg/kg weight for HU-308, AM404 and UCM707. Animals received the first injection of each compound or vehicle 16 h after the local injection of 6-hydroxydopamine. The treatment was repeated daily for a period of 2 weeks post-lesion and the animals were killed 2 h after the last injection. Their brains were rapidly removed, 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, as in our previous study (Lastres-Becker et al., 2005), or 1 week post-lesion, with CBD (3 mg/kg weight) or vehicle during a period of 2 weeks or 1 week, respectively. Then, the animals were killed and their brains removed and processed as described for the above experiments.

4.2.   Neurochemical evaluation of neuronal injury

4.2.1.   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 contents and TH activity as described below.

4.2.2.   Analysis of DA contents
The contents of DA were analyzed using HPLC with electrochemical detection according to our previously published method (González et al., 1999). 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, MA, 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 the 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.

4.2.3.   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 contents, with the only difference of a previous extraction with alumina. Values were expressed as ng of L-dopa formed/area h.

4.3. In situ hybridization techniques

4.3.1. 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.

4.3.2. Analysis of mRNA levels for TH and Cu,Zn-SOD by in situ hybridization
The analysis of mRNA levels for TH and Cu,Zn-SOD was carried out according to procedures previously described (see details in González et al., 2005). 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. For the analysis of TH-mRNA levels, we used a commercial probe (NEN-Du Pont, Itisa, Madrid, Spain; see details in García-Gil et al., 1998), whereas a synthetic 45-base probe, selected from the previously published sequence (5′-TCC AGT CTT TGT ACT TTC TTC ATT TCC ACC TTT GCC CAA GTC ATC-3′; Kunikowska and Jenner, 2001), was used for the analysis of mRNA levels of Cu, Zn-SOD. The specificity of both probes was assessed by Northern Blot analysis. Both were 3′-end labelled with [^{35}S]-dATP using terminal deoxynucleotidyl transferase. Sections were, then, hybridized with [^{35}S]-labelled oligonucleotide probes (7.5×10^5 dpm per section), washed and exposed to X-ray film (βmax, Amersham) for 1 week (TH) or 2 weeks (Cu,Zn-SOD), 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).

4.4. Statistics

All data were assessed by one-way analysis of variance, followed by the Student–Newman–Keuls test.

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