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Sativex-like Combination of Phytocannabinoids is Neuroprotective in Malonate-Lesioned Rats, an Inflammatory Model of Huntington's Disease: Role of CB_1 and CB_2 Receptors

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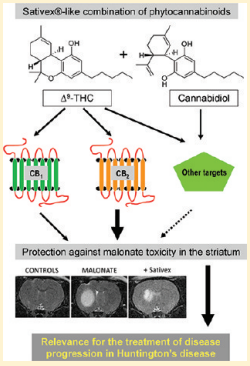
ABSTRACT: We have investigated whether a 1:1 combination of botanical extracts enriched in either Δ^9-tetrahydrocannabinol (Δ^9-THC) or cannabidiol (CBD), which are the main constituents of the cannabis-based medicine Sativex, is neuroprotective in Huntington's disease (HD), using an experimental model of this disease generated by unilateral lesions of the striatum with the mitochondrial complex II inhibitor malonate. This toxin damages striatal neurons by mechanisms that primarily involve apoptosis and microglial activation. We monitored the extent of this damage and the possible preservation of the striatal parenchyma by treatment with a Sativex-like combination of phytocannabinoids using different histological and biochemical markers. Results were as follows: (i) malonate increased the volume of edema measured by in vivo NMR imaging and the Sativex-like combination of phytocannabinoids partially reduced this increase; (ii) malonate reduced the number of Nissl-stained cells, while enhancing the number of degenerating cells stained with FluoroJade-B, and the Sativex-like combination of phytocannabinoids reversed both effects; (iii) malonate caused a strong glial activation (i.e., reactive microglia labeled with Iba-1, and astrogliosis labeled with GFAP) and the Sativex-like combination of phytocannabinoids attenuated both responses; and (iv) malonate increased the expression of inducible nitric oxide synthase and the neurotrophin IGF-1, and both responses were attenuated after the treatment with the Sativex-like combination of phytocannabinoids. We also wanted to establish whether targets within the endocannabinoid system (i.e., CB_1 and CB_2 receptors) are involved in the beneficial effects induced in this model by the Sativex-like combination of phytocannabinoids. This we did using selective antagonists for both receptor types (i.e., SR141716 and AM630) combined with the Sativex-like phytocannabinoid combination. Our results indicated that the effects of this combination are blocked by these antagonists and hence that they do result from an activation of both CB_1 and CB_2 receptors. In summary, this study provides preclinical evidence in support of a beneficial effect of the cannabis-based medicine Sativex as a neuroprotective agent capable of delaying signs of disease progression in a proinflammatory model of HD, which adds to previous data obtained in models priming oxidative mechanisms of striatal injury. However, the interest here is that, in contrast with these previous data, we have now obtained evidence that both CB_1 and CB_2 receptors appear to be involved in the effects produced by a Sativex-like phytocannabinoid combination, thus stressing the broad-spectrum properties of Sativex that may combine activity at the CB_1 and/or CB_2 receptors with cannabinoid receptor-independent actions.



KEYWORDS: *Phytocannabinoids, cannabidiol, Δ^9-tetrahydrocannabinol, CB_1 and CB_2 receptors, Huntington's disease, malonate, basal ganglia, neurodegeneration, neuroprotection*

Huntington's disease (HD) is an inherited neurodegenerative disorder characterized by motor abnormalities, cognitive dysfunction, and psychiatric symptoms.[1] The primary cause of the disease is a mutation in the huntingtin gene consisting of a CAG triplet repeat expansion translated into an abnormal polyglutamine tract in the amino-terminal portion of this protein that becomes toxic for striatal and cortical neuronal subpopulations.[2] At present, there is no specific pharmacotherapy to alleviate motor and cognitive symptoms and/or to arrest/delay disease progression in HD. Thus, even though a few compounds have produced encouraging effects in preclinical studies (i.e., minocycline, coenzyme Q10, unsaturated fatty acids, inhibitors of histone deacetylases) none of the findings obtained in these studies have yet led on to the development of an effective medicine.[3] Importantly, therefore,

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following on from an extensive preclinical evaluation using different experimental models of HD, clinical tests are now being performed with cannabinoids. The preclinical studies with cannabinoids demonstrated preservation of striatal neurons by several agonists against different cytotoxic stimuli that operate in HD pathogenesis,[4,5] effects that were exerted through multiple mechanisms of action, some of which involve the activation of CB_1 and/or CB_2 receptors and others of which do not. For example, cannabinoids with antioxidant profile, that is, Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD), protected striatal neurons against toxicity caused by the mitochondrial complex II inhibitor 3-nitropropionic acid (3NP) that primes oxidative injury.[6,7] However, the activity of Δ^9-THC at the CB_1 and CB_2 receptors enables this phytocannabinoid to induce neuroprotection in other experimental models, for example, the transgenic mouse model of HD, R6/2, in which the effects of Δ^9-THC are likely produced through the activation of CB_1 receptors[8] and possibly through the activation of CB_2 receptors too, as selective agonists of this receptor type preserved striatal neurons in this genetic model[9] and also in malonate-lesioned rats,[10] a model priming pro-inflammatory events. Selective agonists of CB_1[8,11] and CB_2[9] receptors also preserved striatal neurons in in vitro or in vivo excitotoxic models. By contrast, other studies showed no effects in R6/1 mice using Δ^9-THC, the synthetic agonist HU-210 and the inhibitor of the endocannabinoid metabolism URB597.[12] All these data suggest that the evaluation of cannabinoids as disease-modifying agents in patients should be necessarily conducted with a broad-spectrum cannabinoid or with combinations of various cannabinoids with different and complementary pharmacological profiles. Sativex, a cannabis-based medicine recently licensed for the treatment of spasticity and pain in multiple sclerosis patients,[13,14] has an appropriate profile for HD as it can activate CB_1 and CB_2 receptors due to the presence of Δ^9-THC, but it can also exert cannabinoid receptor-independent antioxidant properties due to Δ^9-THC and, in particular, to CBD.[15] We recently initiated some experiments with the combination of Δ^9-THC and CBD botanical extracts present in Sativex in those animal models of HD, in which individual cannabinoid agonists have proved to be effective,[6−11] with the objective of determining whether this mixture also works in these models. We have just published the first data obtained in rats subjected to 3NP intoxication,[16] a model priming, as mentioned above, calpain activation and oxidative injury as major cytotoxic mechanisms, and in which pure Δ^9-THC[6] and CBD[7] administered separately, have already been found to display neuroprotective properties. The Sativex-like combination of phytocannabinoids also preserved striatal neurons from death caused by 3NP intoxication, and this effect was, as expected, independent of CB_1 and CB_2 receptors.[16] These findings prompted us to extend our research to rats lesioned with malonate, an acute model of HD in which striatal damage is produced primarily by apoptosis and glial activation/inflammatory events and in which selective CB_2 receptor agonists have been shown to be effective.[10] To this end, we lesioned rats with an intrastriatal injection of malonate and used these animals for two different sets of experiments. First, we examined the neuroprotective effects of a 1:1 combination of botanical extracts of Δ^9-THC and CBD, a combination that approximates to the 1.0:0.93 mixture of these extracts that is present in Sativex. The level of neuroprotection was evaluated by measuring the following parameters: (i) the volume of edema measured by in vivo NMR imaging; (ii) the number of Nissl- and FluoroJade B-stained cells which correlate with the number of surviving and degenerating cells, respectively, in the striatal parenchyma; (iii) the presence of reactive microgliosis, by using Iba-1 immunohistochemistry, and astrogliosis labeled with GFAP immunostaining; and (iv) the expression of various biochemical markers that have been found previously to be altered in this and other HD models, i.e. inducible nitric oxide synthase (iNOS), the neurotrophin IGF-1 and the CB_1 receptor.[8,10,16,17] In the second set of experiments, we explored whether the neuroprotective effects observed in this model with the combination of Δ^9-THC and CBD present in Sativex involved the activation of CB_1 receptors, CB_2 receptors, or both, using selective antagonists for these two receptors (SR141716 and AM630, respectively). For this second set of experiments, we monitored the number of Nissl-stained cells, since this constitutes a very selective and sensitive marker of malonate damage in the striatal parenchyma.

RESULTS AND DISCUSSION

Only a few clinical studies have been performed to determine whether cannabinoid compounds are efficacious in HD, and the results they have yielded are rather controversial.[18−21] Possibly, the reason for such controversy is that these clinical studies concentrated more on HD symptoms rather than on disease progression. Recent animal studies, however, have demonstrated that combinations of different cannabinoids or the use of a broad-spectrum cannabinoid may delay disease progression by preserving striatal neurons from death in different animal models of HD, thus stressing again the need for new clinical studies directed now at testing whether the neuroprotective effects that certain cannabinoids induce in experimental models of HD[8−11,16] are also produced in HD patients. As mentioned in the introduction, we recently initiated some experiments with a 1:1 combination of the Δ^9-THC and CBD botanical extracts that are present in Sativex in those animal models of HD, in which individual cannabinoid agonists have been found to be protective,[6−11] with the objective to determine whether this mixture also works in these models. We used first rats subjected to 3NP intoxication, a model priming calpain activation and oxidative injury as major cytotoxic mechanisms, and in which pure Δ^9-THC[6] and pure CBD[7] administered by themselves, had displayed neuroprotective properties. The Sativex-like combination of phytocannabinoids also protected striatal neurons from death caused by 3NP intoxication and its effects were, as expected, independent of CB_1 and CB_2 receptors.[16] In this Article,[16] we already presented preliminary evidence that the Sativex-like combination of phytocannabinoids might also work in additional models of HD, as it was capable to attenuate malonate-induced iNOS up-regulation in rats lesioned with this neurotoxin, but this was the only parameter analyzed in these rats.[16] The goal of the present study was to corroborate the potential of Sativex-like combination of phytocannabinoids in this model of HD, in which striatal damage is primarily produced by apoptosis and glial activation/inflammatory events,[10] by measuring different histopathological and biochemical parameters that have been more directly related to striatal degeneration in this HD model.

Effects of Phytocannabinoids on Malonate-Induced Striatal Damage. The first objective was to establish that we could produce the characteristic striatal damage associated with the local administration of malonate.[10] As expected, the administration of malonate damaged the striatal parenchyma as indicated by the increased edema volume recorded in in vivo

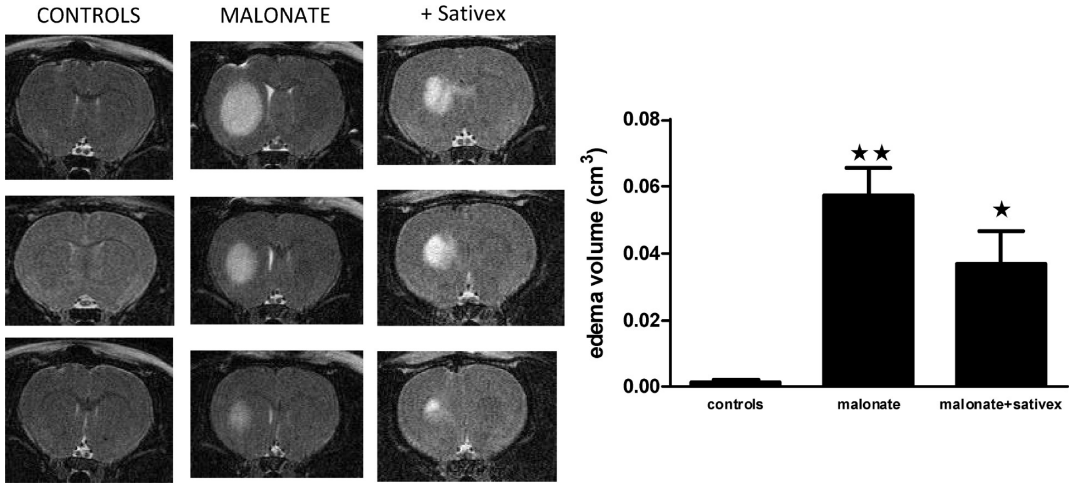

Figure 1. Volume of edema measured by in vivo NMR imaging procedures in the caudate-putamen of rats subjected to unilateral lesions with malonate and receiving two injections of Δ^9-THC- and CBD-enriched botanical extracts combined in a Sativex-like ratio 1:1 (total cannabinoid dose equivalent to 3 mg/kg weight), or vehicle (Tween 80-saline). Details in the text. The 1:1 mixture Δ^9-THC- and CBD-enriched botanical extracts is referred to as Sativex in this figure. Values are expressed as means ± SEM for 5−6 animals per group. Data were subjected to one-way analysis of variance followed by the Student−Newman−Keuls test (*p < 0.05, **p < 0.005 compared with controls). Representative in vivo NMR images for each experimental group are included in the left panel.

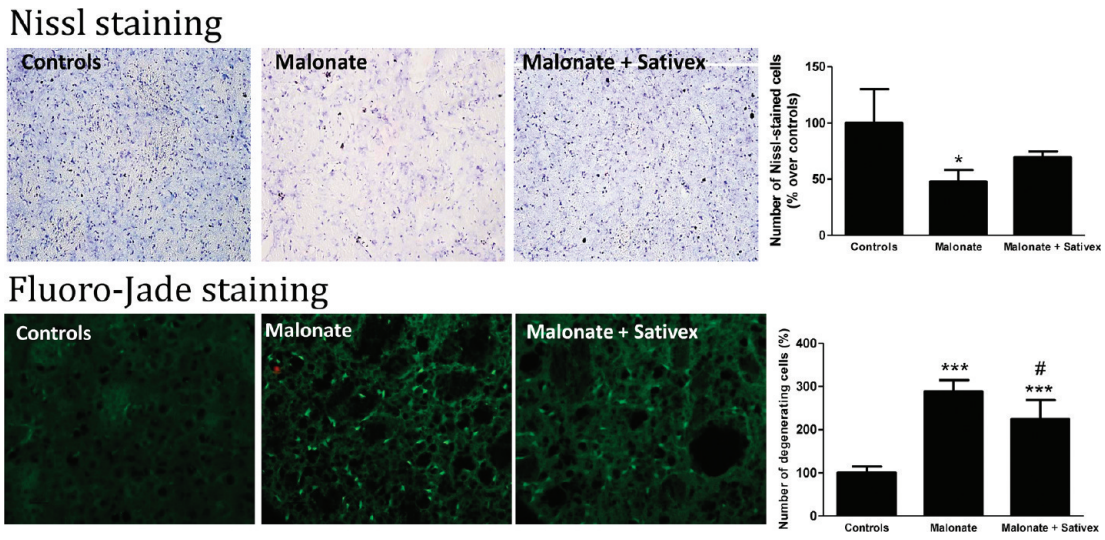

Figure 2. Nissl and FluoroJade B staining measured in the caudate-putamen of rats subjected to unilateral lesions with malonate and receiving two injections of Δ^9-THC- and CBD-enriched botanical extracts combined in a Sativex-like ratio 1:1 (total cannabinoid dose equivalent to 3 mg/kg weight), or vehicle (Tween 80-saline). Details in the text. The 1:1 mixture Δ^9-THC- and CBD-enriched botanical extracts is referred to as Sativex in this figure. Values correspond to percent over the contralateral nonlesioned side and are expressed as means ± SEM for 5−6 animals per group. Data were subjected to one-way analysis of variance followed by the Student−Newman−Keuls test (*p < 0.05, **p < 0.01, ***p < 0.005 compared with controls; $^{\#}p$ < 0.05 compared with malonate + vehicle). Representative Nissl- and FluoroJade B-stained microphotographs for each experimental group are included in the left panel. Magnification = 20×.

NMR imaging ($F(2,19) = 8.86$, $p < 0.005$; see Figure 1), the reduction in the number of surviving cells determined by Nissl staining ($F(2,10) = 6.36$, $p < 0.05$; see Figure 2), the increase in the level of degenerating cells determined by FluoroJade B staining ($F(2,10) = 32.87$, $p < 0.0005$; see Figure 2), and the extent of reactive microgliosis labeled with Iba-1 immunohistochemistry ($F(2,12) = 4.652$, $p < 0.05$; see Figure 3) and astrogliosis labeled with GFAP immunohistochemistry ($F(2,11) = 9.25$, $p < 0.01$; see Figure 3). Malonate toxicity also affects the expression of different markers related to inflammation (i.e., iNOS: $F(2,11) = 5.78$, $p < 0.05$) and neurotrophins (i.e., IGF-1: $F(2,15) = 4.41$, $p < 0.05$) (see Figure 4). These changes, although characteristic of a specific experimental model of HD, have also been found in HD patients using CSF, blood cell, and post-mortem brain samples,[22,23] thus supporting the idea that glial activation associated with inflammatory events is part in the pathogenic process that occurs in HD patients. Lastly, malonate toxicity is also associated with a reduction in the expression of CB_1 receptors ($F(2,25) = 4.863$, $p < 0.05$; see Figure 4) an effect largely related to HD pathogenesis in different animal models.[6,8,16]

The administration of a 1:1 combination of Δ^9-THC- and CBD-enriched botanical extracts that are present in Sativex attenuated, to a different extent, all these malonate-induced changes. For example, it slightly reduced the volume of edema measured with in vivo NMR imaging procedures 24 h after

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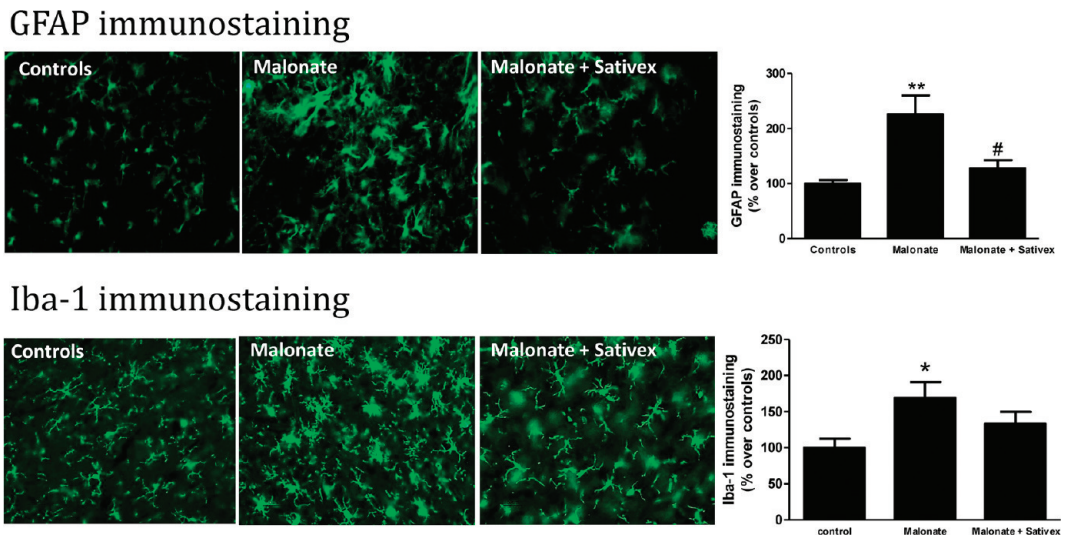

Figure 3. Iba-1 and GFAP immunostainings measured in the caudate-putamen of rats subjected to unilateral lesions with malonate and receiving two injections of Δ^9-THC- and CBD-enriched botanical extracts combined in a Sativex-like ratio 1:1 (total cannabinoid dose equivalent to 3 mg/kg weight), or vehicle (Tween 80-saline). Details in the text. The 1:1 mixture Δ^9-THC- and CBD-enriched botanical extracts is referred to as Sativex in this figure. Values correspond to percent over the contralateral nonlesioned side and are expressed as means ± SEM for 3−4 animals per group. Data were subjected to one-way analysis of variance followed by the Student−Newman−Keuls test ($^*p < 0.05$, $^{**}p < 0.01$ compared with controls; $^{\#}p < 0.05$ compared with malonate + vehicle). Representative Iba-1 and GFAP immunostained microphotographs for each experimental group are included in the left panel. Magnification = 20×.

malonate application and 24 h before animal death (Figure 1). These in vivo results correlated well with the data obtained after the histopathological analysis of animal brains. For example, the treatment with a Sativex-like combination of phytocannabinoids increased the number of Nissl-stained cells that had been reduced by malonate (see Figure 2), while reducing the high number of degenerating cells stained with FluoroJade-B (see Figure 2). In a similar way, the Sativex-like combination of phytocannabinoids attenuated the activation of glial elements caused by malonate (i.e., reactive microglia labeled with Iba-1, and astrogliosis labeled with GFAP; see Figure 3), which was particularly abundant and generalized along the whole striatal parenchyma in this experimental model. Lastly, the Sativex-like combination of phytocannabinoids attenuated the malonate-induced up-regulatory responses in iNOS (already described in ref 16) and IGF-1 gene expression in the striatal parenchyma (see Figure 4), whereas it partially recovered the malonate-induced reduction in CB_1 receptors (see Figure 4). These findings support the notion that the combination of both phytocannabinoid-botanical extracts may be effective as a neuroprotective therapy in malonate-lesioned rats as it was previously found when individual cannabinoid agonists (i.e., HU-308) were used in the same HD model.[10] In this study,[10] however, we found that CBD administered alone was not active against malonate-induced striatal damage, but, according to the present data, its combination with Δ^9-THC resulted in highly positive effects presumably by enhancing the activity of this last phytocannabinoid at the CB_1 and CB_2 receptors (see below). The rationale for such combination has been largely discussed in previous reviews.[24]

Involvement of CB_1 and/or CB_2 Receptors in the Beneficial Effects of Phytocannabinoids on Malonate-Induced Striatal Damage. In our previous study that used an experimental model of HD that primes oxidative injury and calpain activation as major causes of striatal damage, we detected protective effects of the Sativex-like combination of phytocannabinoids, that seemed to result from the antioxidant and cannabinoid receptor-independent properties of both phytocannabinoids rather than from any interaction with CB_1 and/or CB_2 receptors. We wanted to see if this was also the case in the experimental model of HD used in the present study. To this end, we conducted additional experiments with selective antagonists for CB_1 and CB_2 receptor types, that is, SR141716 and AM630, respectively, combined with the Sativex-like mixture of phytocannabinoids. The same or similar antagonists had been already tested after being administered alone in the same animal model of HD, showing no effect in the case of CB_2 receptor blockade[10] or even aggravating striatal damage in the case of SR141716.[25] In our present study, the blockade of CB_1 receptors with SR141716 or the blockade of CB_2 receptors with AM630 reversed the phytocannabinoid-induced decrease in the ability of malonate to attenuate Nissl-staining of cells ($F(5,23) = 6.11$, $p < 0.005$; see Figure 5). Interestingly, this reversal was no greater when both antagonists were administered together (see Figure 5). However, it is important to note that, although SR141716 and AM630, both separately and together, reversed the effects of the Sativex-like combination of Δ^9-THC- and CBD-enriched botanical extracts, the lowest number of Nissl-stained cells was observed after the treatment with AM630, administered alone or in combination with SR141716. This finding, together with our previously published data showing that CB_2 receptors underwent an up-regulation in glial elements located in the striatal parenchyma after malonate,[10] whereas CB_1 receptors underwent a down-regulation in the same animal model in the present study (see Figure 4) and also in other models of HD,[8,16] suggests that even though Δ^9-THC can activate both CB_1 and CB_2 receptors, the CB_2 receptor plays a greater role than the CB_1 receptor in the protection from malonate-induced striatal damage that the Δ^9-THC- and CBD-enriched botanical extracts produce.

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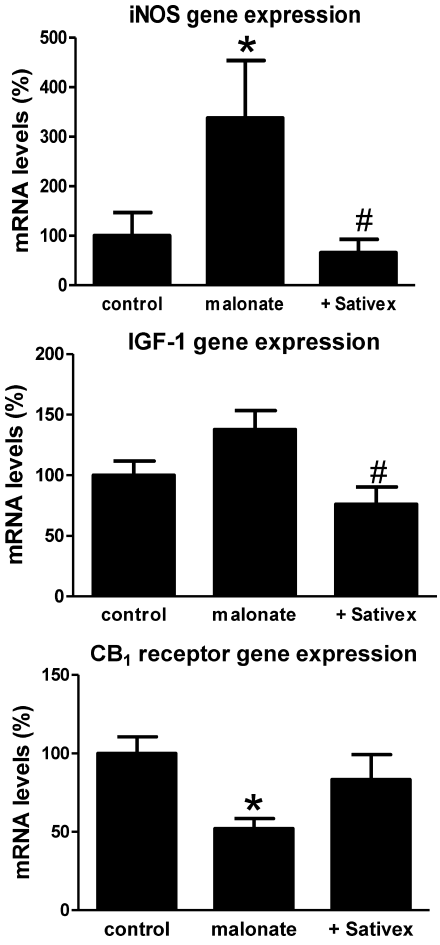

Figure 4. Gene expression for iNOS, IGF-1, and CB_1 receptors measured in the caudate-putamen of rats subjected to unilateral lesions with malonate and receiving two injections of Δ^9-THC- and CBD-enriched botanical extracts combined in a Sativex-like ratio 1:1 (total cannabinoid dose equivalent to 3 mg/kg weight), or vehicle (Tween 80-saline). Details in the text. The 1:1 mixture Δ^9-THC- and CBD-enriched botanical extracts is referred to as Sativex in this figure. Values correspond to percent over the contralateral nonlesioned side and are expressed as means ± SEM for 5−6 animals per group. Data were subjected to one-way analysis of variance followed by the Student−Newman−Keuls test (*p < 0.05 compared with controls; #p < 0.05 compared with malonate + vehicle).

CONCLUSION

Collectively, this study provides preclinical evidence in support of a beneficial effect of the cannabis-based medicine Sativex as a neuroprotective agent capable of delaying disease progression in a proinflammatory model of HD, which adds to previous data obtained in models priming oxidative mechanisms of striatal injury. However, the interest here is that, in contrast to these previous data, we have now obtained evidence that both CB_1 and CB_2 receptors are likely to be involved in Sativex effects, thus stressing the broad-spectrum properties of this medicine that may combine activity at the CB_1 and/or CB_2 receptors with cannabinoid receptor-independent actions. Moreover, our previous data showing that, in this proinflammatory model of HD, CB_2 receptors were up-regulated[10] whereas CB_1 receptors were down-regulated (also demonstrated here), as well as our observation that the protective effect of the Sativex-like combination of Δ^9-THC- and CBD-enriched botanical extracts could be blocked by the selective CB_2 receptor antagonist AM630, suggest that CB_2 receptors play a particularly important role in this protective effect, a hypothesis that is also in concordance with the previous literature (see ref 26 for review). Lastly, given that HD is a disorder that is currently poorly managed in the clinic, the present data obtained with malonate-lesioned rats, together with previously published data obtained using an oxidative model of HD,[16] provide a strong justification for mounting clinical trials with cannabinoids that produce positive effects in these preclinical models of HD.

MATERIALS AND METHODS

Animals and Surgery. Adult (12 week old; 350−400 g) male Sprague−Dawley rats (Harlan Ibérica, Barcelona, Spain) were used for the experiments. Animals were housed in a room with controlled photoperiod (08:00−20:00 light) and temperature (22 ± 1 °C) with free access to standard food and water. All experiments were conducted according to local and European rules (directive 86/609/EEC) and approved by the "Comité de Experimentación Animal" of our university. Rats were subjected to unilateral lesions of the striatum with the complex II inhibitor malonate, following a procedure described previously.[10] Rats were injected stereotaxically (coordinates: +0.8 mm anterior, +2.9 mm lateral from the bregma, −4.5 mm ventral from the dura mater) into the left striatum with 2 M malonate (dissolved in PBS 0.1 M, pH = 7.4) in a volume of 1 μL. The contralateral striatum of each animal remained unaffected, when data generated in this experimental model are expressed as percent of the lesioned side over the corresponding nonlesioned side.

Treatments and Sampling. We followed the same procedure used in previous studies with cannabinoids in malonate-lesioned rats.[10,25] Thus, 30 min before and 2 h after the intrastriatal injection of malonate, animals were treated with combinations of botanical extracts enriched with either Δ^9-THC, kindly provided by GW Pharmaceuticals Ltd., Cambridgeshire, U.K. (Δ^9-THC botanical extract contains 67.1% Δ^9-THC, 0.3% CBD, 0.9% cannabigerol, 0.9% cannabichromene, and 1.9% other phytocannabinoids) or CBD, also provided by GW Pharmaceuticals Ltd., Cambridgeshire, U.K. (CBD botanical extract contains 64.8% CBD, 2.3% Δ^9-THC, 1.1% cannabigerol, 3.0% cannabichromene, and 1.5% other phytocannabinoids). The total dose of cannabinoid administered was always 4.63 mg/kg (equivalent to 3 mg/kg of pure CBD + Δ^9-THC), a dose within the range of effective doses of both compounds when they were administered in pure form in this and other experimental models of HD.[8−11] This was also the effective dose in our previous study with Sativex-like combination of phytocannabinoids in another HD model.[16] Cannabinoids were prepared in Tween 80-saline solution (1:16) and they were administered i.p. Malonate-lesioned rats administered with vehicle, as well as sham-operated animals, were also included in this experiment. In a further experiment, rats injected with malonate, following the same procedure described above, and also injected with the 1:1 combination of Δ^9-THC- and CBD-enriched botanical extracts used in the previous experiment, were coadministered (10 min before each injection of Sativex-like combination) with the CB_1 receptor antagonist SR141716 (1 mg/kg), kindly provided by Sanofi-Aventis (Montpellier, France) or the CB_2 receptor blocker AM630 (1 mg/kg), purchased from Tocris (Biogen Científica, Madrid, Spain), both prepared in Tween 80-saline (1:16). In both experiments, animals were killed 48 h after the administration of malonate and their brains were rapidly removed, the two striata dissected and frozen separately in 2-methylbutane cooled in dry ice, and stored at −80 °C for subsequent qRT-PCR analysis. Animals to be used for histological analysis were transcardially perfused with saline followed by fresh 4% paraformaldehyde [in 0.1 M phosphate buffered-saline (PBS)], and their brains were collected and postfixed overnight at 4 °C, and then immersed in antifreeze solution and stored at −20 °C for Nissl and FluoroJade B staining and immunohistochemical analysis. In all experiments, at least 5−6 animals were used per experimental group.

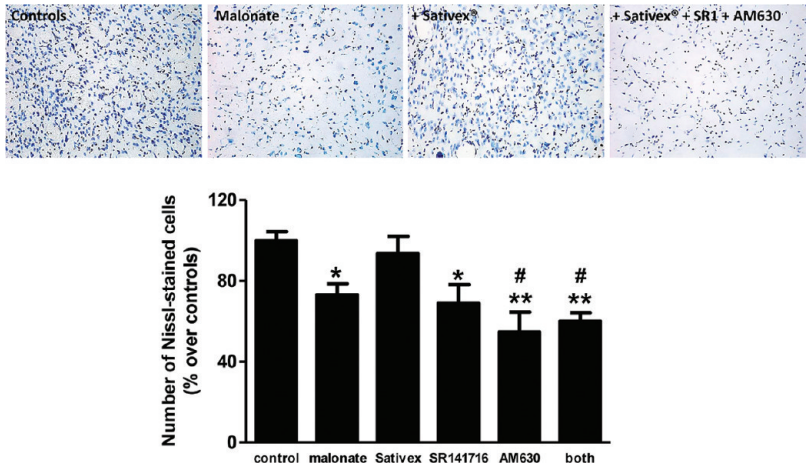

Figure 5. Nissl staining measured in the caudate-putamen of rats subjected to unilateral lesions with malonate and receiving two injections of Δ^9-THC- and CBD-enriched botanical extracts combined in a Sativex-like ratio 1:1 (total cannabinoid dose equivalent to 3 mg/kg weight), selective antagonists for the CB_1 (SR141716) and/or the CB_2 (AM630) receptor, or vehicle (Tween 80-saline). Details in the text. The 1:1 mixture Δ^9-THC- and CBD-enriched botanical extracts is referred to as Sativex in this Figure. Values correspond to percent over the control group and are expressed as means ± SEM for 5−6 animals per group. Data were subjected to one-way analysis of variance followed by the Student−Newman−Keuls test (*p < 0.05, **p < 0.01 compared with controls; $^{\#}p$ < 0.05 compared with malonate + Sativex).

In Vivo NMR Imaging Procedures. In the first experiment consisting of injections of Sativex-like combinations of phytocannabinoids to malonate-lesioned rats, animals from the three experimental groups were used, at 24 h after malonate lesion and 24 h before animal death, for in vivo NMR imaging studies in which the volume of striatal edema was calculated. Experiments were performed at the Nuclear Magnetic Resonance Centre of Complutense University (Madrid, Spain) using a BIOSPEC BMT 47/40 (Bruker, Ettlingen, Germany) operating at 4.7 T, equipped with a 12 cm actively shielded gradient system. Rats were anesthetized with oxygen:isoflurane and subsequently placed in prone position inside a cradle. The animal head was immobilized and placed underneath a 4 cm surface coil. A respiration sensor was used to control the animals. First global shimmer was assessed, and then three gradient-echo scout images in axial, sagittal, and coronal directions were acquired (TR/TE = 100/3.2 ms, matrix = 128 × 128). A 3D fast spin−echo experiment with axial slice orientation was subsequently performed using the following acquisition parameters: TR = 3000 ms, effective TE = 86.5 ms, NA = 2, FOV = 2.56 × 2.56 × 1.28 cm^3, matrix size = 256 × 128 × 32. The reconstructed matrix size was 256 × 256 × 32. The total time of the acquisition experiment was 27 min.

Histological Analyses. Coronal brain sections (25 μm thick) were obtained with a vibratome and collected as floating slices at the level of the caudate-putamen. They were used for Nissl (see details in ref 27) and FluoroJade B (see details in ref 10) staining, which permitted determination of the effects of particular treatments on cell number, and for immunohistochemical analysis of Iba-1, a marker of microglial cells, and GFAP, a marker of astrocytes. For immunohistochemistry, sections were incubated overnight at 30 °C with (i) monoclonal antirat Iba-1 antibody (Wako, Neuss, Germany) used at 1:300, or (ii) monoclonal antirat GFAP (Sigma-Aldrich, Madrid, Spain) used at 1:400. After incubation with the corresponding primary antibody, sections were washed in 0.1 M PBS and incubated for 2 h at 37 °C with a mouse highly cross-adsorbed AlexaFluor 488 secondary antibody (Invitrogen, Carlsbad, CA) for GFAP immunostaining, and with a rabbit biotinylated secondary antibody (Sigma-Aldrich, Madrid, Spain) followed by incubation for 2 additional hours with streptavidin 488 (Molecular Probes, Paisley, U.K.) for Iba-1 immunostaining. Both secondary antibodies were used at 1:200. Negative control sections were obtained using the same protocol with omission of the primary antibody. All sections for each immunohistochemical procedure were processed at the same time and under the same conditions. A Nikon Eclipse 90i confocal microscope and a Nikon DXM 1200F camera were used for slide observation and photography, and all image processing, including cell counting, was done using ImageJ, the software developed and freely distributed by the U.S. National Institutes of Health (Bethesda, MD). For this purpose, multiple sections, selected from levels located approximately 200 μm from the middle of the lesion, were obtained from each brain and used to generate a mean value per subject.

Real Time qRT-PCR Analysis. Total RNA was isolated from striata using RNATidy reagent (AppliChem. Inc., Cheshire, CT). The total amount of RNA extracted was quantitated by spectrometry at 260 nm and its purity from the ratio between the absorbance values at 260 and 280 nm, whereas its integrity was confirmed in agarose geles. After genomic DNA was removed (to eliminate DNA contamination), single-stranded cDNA was synthesized from 1 μg of total RNA using a commercial kit (Rneasy Mini Quantitect Reverse Transcription, Qiagen, Izasa, Madrid, Spain). The reaction mixture was kept frozen at −20 °C until enzymatic amplification. Quantitative RT-PCR assays were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) to quantify mRNA levels for IGF-1 (ref Rn99999087_m1), iNOS (ref Rn00561646_m1), or CB_1 receptor (ref Rn00562880_m1), using β-actin expression (ref Rn00667869_m1) as an endogenous control gene for normalization. The PCR assay was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA), and the threshold cycle was calculated by the instrument's software (7300 Fast System, Applied Biosystems, Foster City, CA).

Statistics. Data were assessed by one-way ANOVA, followed by the Student−Newman−Keuls test.

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Author Contributions

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Author Contributions

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Author Contributions

O.S., R.G.P., and J.F.-R. designed the experiments included in this Article, which were conducted by S.V. and V.S. and supervised by O.S. and J.F.-R. J.F.-R. wrote the different versions of the manuscript, which were revised by the other

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Notes

The authors declare the following competing financial interest(s):Authors have formal links with GW Pharmaceuticals that funds some of their research.

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■ ABBREVIATIONS

3-NP, 3-nitropropionate; CBD, cannabidiol; GFAP, glial fibrillary acidic protein; HD, Huntington's disease; IGF-1, insulin like growth factor-1; iNOS, inducible nitric oxide synthase; PBS, phosphate buffer saline; Δ^9-THC, Δ^9-tetrahydrocannabinol

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