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# Neuroprotective Effects of Phytocannabinoid-Based Medicines in Experimental Models of Huntington's Disease

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We studied whether combinations of botanical extracts enriched in either $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) or cannabidiol (CBD), which are the main constituents of the cannabis-based medicine Sativex, provide neuroprotection in rat models of Huntington's disease (HD). We used rats intoxicated with 3-nitropropionate (3NP) that were given combinations of $\Delta^9$-THC- and CBD-enriched botanical extracts. The issue was also studied in malonate-lesioned rats. The administration of $\Delta^9$-THC- and CBD-enriched botanical extracts combined in a ratio of 1:1 as in Sativex attenuated 3NP-induced GABA deficiency, loss of Nissl-stained neurons, down-regulation of $CB_1$ receptor and IGF-1 expression, and up-regulation of calpain expression, whereas it completely reversed the reduction in superoxide dismutase-1 expression. Similar responses were generally found with other combinations of $\Delta^9$-THC- and CBD-enriched botanical extracts, suggesting that these effects are probably related to the antioxidant and $CB_1$ and $CB_2$ receptor-independent properties of both phytocannabinoids. In fact, selective antagonists for both receptor types, i.e., SR141716 and AM630, respectively, were unable to prevent the positive effects on calpain expression caused in 3NP-intoxicated rats by the 1:1 combination of $\Delta^9$-THC and CBD. Finally, this combination also reversed the up-regulation of proinflammatory markers such as inducible nitric oxide synthase observed in malonate-lesioned rats. In conclusion, 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 HD, a disorder that is currently poorly managed in the clinic, prompting an urgent need for clinical trials with agents showing positive results in preclinical studies. 

**Key words:** phytocannabinoids; cannabidiol; $\Delta^9$-tetrahydrocannabinol; cannabinoid receptors; Huntington's disease; 3-nitropropionic acid; malonate; basal ganglia; neurodegeneration; neuroprotection

Various cannabinoid compounds have been reported to attenuate brain damage caused by different type of insults and reproduced in a variety of in vitro and in vivo models of acute or chronic neurodegeneration (for review see Fernández-Ruiz et al., 2010). These effects are produced by the capability of cannabinoids to control multiple mechanisms involved in neuronal death, including excitotoxicity (exerted mainly through the activation of $CB_1$ receptors; see van der Stelt et al., 2002), inflammatory events (exerted mainly through the activation of $CB_2$ receptors; see Fernández-Ruiz et al., 2007), and oxidative injury (exerted presumably through cannabinoid receptor-independent mechanisms; see Fernández-Ruiz et al., 2005). These findings, together with

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the observation that certain elements of the cannabinoid signaling system, in particular, the endocannabinoids anandamide and 2-arachidonoyl-glycerol and the $CB_2$ receptor, are significantly up-regulated in response to brain damage, support the idea that the cannabinoid system might play a key protective role in response to excitotoxic, inflammatory, traumatic, or oxidative injury (for review see Fernández-Ruiz et al., 2010).

Huntington's disease (HD) is one of the disorders for which certain cannabinoid compounds have been found to have neuroprotective properties in experimental models. HD is an inherited neurodegenerative disorder characterized by motor abnormalities, cognitive dysfunction, and psychiatric symptoms that are produced by selective lesions in the cerebral cortex and, in particular, in the striatum (for recent review see Roze et al., 2010). The lesions are caused by a mutation in the gene encoding the protein huntingtin, that consists of a CAG triplet repeat expansion translated into an abnormal polyglutamine tract in the amino-terminal portion of this protein (Zucato et al., 2010). However, the intimate mechanisms underlying mutated huntingtin toxicity and striatal and cortical degeneration are still unknown. At present, there is no specific pharmacotherapy to alleviate symptoms and/or to arrest or delay striatal degeneration in HD, although a few compounds (i.e., minocycline, coenzyme Q10, unsaturated fatty acids, inhibitors of histone deacetylases) are presently under clinical evaluation (Johnson and Davidson, 2010). Cannabinoids are also close to clinical testing based on solid preclinical evidence obtained in various experimental models of HD, in which specific cannabinoid agonists have provided positive results for protection against striatal degeneration (for review see Pazos et al., 2008; Fernández-Ruiz et al., 2010). Thus, antioxidant cannabinoids, including the phytocannabinoids $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) and cannabidiol (CBD), protected striatal neurons against toxicity caused by 3-nitropropionic acid (3NP; Lastres-Becker et al., 2004; Sagredo et al., 2007). This is an inhibitor of mitochondrial complex II frequently used to generate striatal atrophy (Brouillet et al., 1999, 2005) reminiscent of HD and of the characteristic deficiency in this complex described in HD patients (Damiano et al., 2010). $\Delta^9$-THC was also neuroprotective in a transgenic mouse model of HD, R6/2, most likely acting through the activation of $CB_1$ receptors (Blázquez et al., 2011). Selective agonists of the $CB_2$ receptors were also protective both in this genetic model (Palazuelos et al., 2009) and in other experimental models in which inflammatory events are predominant (e.g., malonate toxicity; see Sagredo et al., 2009). $CB_1$ (Pintor et al., 2006) and $CB_2$ (Palazuelos et al., 2009) receptor agonists were also protective in excitotoxic models (e.g., quinolinate-lesioned rodents), whereas $CB_1$ receptor agonists reduced cell death induced by excitotoxicity in a cellular model of HD (Blázquez et al., 2011). The fact that multiple targets ($CB_1$ and $CB_2$ receptors) and different cannabinoid compounds have been linked to neuroprotection in experimental models of HD suggests that, when the issue is going to be studied in patients, experiments should be done with a broad-spectrum cannabinoid or with combinations of various cannabinoids with different profiles. One promising option is Sativex, a cannabis-based medicine recently licenced for the treatment of spasticity and pain in multiple sclerosis patients (Russo and Guy, 2006; Wright, 2007). Sativex is an equimolecular combination of $\Delta^9$-THC- and CBD-enriched botanical extracts that may act through different mechanisms/targets, mainly by antioxidant mechanisms in the case of both phytocannabinoids and by activation of $CB_1$ and $CB_2$ receptors in the case of $\Delta^9$-THC (Pertwee, 2009). Therefore, it has a pharmacological profile that appears promising for HD. However, before proceeding to a clinical evaluation of this medicine in HD, it would be interesting to determine whether the combination of $\Delta^9$-THC and CBD botanical extracts used for Sativex also works in animal models of HD. For this purpose, we used the same HD model, rats subjected to 3NP intoxication, in which both $\Delta^9$-THC (Lastres-Becker et al., 2004) and CBD (Sagredo et al., 2007) administered as pure and individual compounds have already been found to display neuroprotective properties. We conducted three experiments in these animals. First, we examined the neuroprotective effects of a 1:1 combination of botanical extracts of $\Delta^9$-THC and CBD, the same as that used in Sativex. The degree of neuroprotection was evaluated by measuring the extent of GABA deficiency and the number of Nissl-stained neurons in the striatal parenchyma as well as the expression of a series of markers related to 3NP intoxication and/or HD pathology. These markers were 1) the antioxidant enzyme superoxide dismutase-1 (SOD-1; see Sagredo et al., 2007), 2) the calcium-binding protein calpain (Bizat et al., 2003a,b; Brouillet et al., 2005), and 3) the neurotrophin insulin-like growth factor-1 (IGF-1; Alexi et al., 1999; Mochel et al., 2007; Pouladi et al., 2010). We also measured the expression of the $CB_1$ receptor, because previously published data suggest that this may serve as a marker of striatal degeneration in patients and experimental models of HD (Blázquez et al., 2011). Several inflammatory markers, e.g., OX-42 and GFAP immunostaining and expression of inducible nitric oxide synthase (iNOS), were also employed, even though that inflammation is very modest in this model, which relies more on oxidative events. We also carried out a few analyses (calpain and SOD-1 expression, which are significantly affected in 3NP intoxicated rats) in experiments conducted with two additional combinations of $\Delta^9$-THC and CBD botanical extracts, i.e., 1:2 and 2:1, with the total cannabinoid administered always equivalent to 3 mg/kg. In a third experiment, we explored whether the neuroprotective effects observed with the combination of $\Delta^9$-THC and CBD used for Sativex were induced by the activation of $CB_1$ or $CB_2$ receptors using selective antagonists for these two receptors (SR141716 and AM630, respectively). For this third experiment, we recorded expression of calpain, which constitutes a very selective and sensitive marker of 3NP intoxication (Bizat et al., 2003a,b; Brouillet et al., 2005). Finally, we also wanted to study whether the Sativex-like combination might also afford neuroprotection in another experimental model of HD. To this end, we

lesioned rats using intrastriatal injection of malonate, which, as mentioned above, generates striatal damage associated with a marked up-regulation of inflammatory markers such as iNOS (Sagredo et al., 2009).

## MATERIALS AND METHODS

### Animals, Treatments, and Sampling

Adult (12 weeks 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°C ± 1°C) with free access to standard food and water. All experiments were conducted according to local and European rules (directive 86/609/EEC). Rats were subjected to intraperitoneal (i.p.) injections of 3NP for 5 days (two injections per day) at a dose of 10 mg/kg each. At 4 hr after the first injection of 3NP, rats were also injected with combinations of botanical extracts enriched with either $\Delta^9$-THC, kindly provided by GW Pharmaceuticals Ltd. (Cambridgeshire, United Kingdom; $\Delta^9$-THC botanical extract contains 67.1% $\Delta^9$-THC, 0.3% CBD, 0.9% cannabigerol, 0.9% cannabichromene, and 1.9% other phytocannabinoids) or CBD, also provided by GW Pharmaceuticals Ltd. (CBD botanical extract contains 64.8% CBD, 2.3% $\Delta^9$-THC, 1.1% cannabigerol, 3.0% cannabichromene, and 1.5% other phytocannabinoids). We focused mainly on the 1:1 combination used in Sativex, but we also conducted additional experiments with the 1:2 and 2:1 combinations, although the total dose of cannabinoid administered was always 4.63 mg/kg (equivalent to 3 mg/kg of pure CBD and $\Delta^9$-THC), a dose within the range of effective doses of both compounds when they were administered in pure form in this experimental HD model (Lastres-Becker et al., 2004; Sagredo et al., 2007). Cannabinoids were prepared in Tween 80-saline solution (1:16) and were administered i.p.; 3NP-lesioned rats treated with vehicle (Tween 80-saline) were also included in this experiment. Injections were repeated daily for a total period of 5 days, and the animals were killed 90 min 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 and histological evaluation indicative of the degree of 3NP-induced neuronal injury. In the case of samples that were used for qRT-PCR analysis, the two striata of each animal were dissected immediately after killing and were frozen at –80°C. In a further experiment, rats injected with 3NP, by the same procedure described above, were injected once daily over 5 days with the 1:1 combination of $\Delta^9$-THC- and CBD-enriched botanical extracts used in the previous experiment, administered alone or coadministered (15 min before Sativex-like combination) daily 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 S.L., Madrid, Spain), both prepared in Tween 80-saline (1:16). Animals were killed 90 min after the last injection, and their brains were rapidly removed and processed as described above.

We also used a few rats for inducing unilateral lesions of the striatum with malonate, another complex II inhibitor, following a procedure described previously (Sagredo et al., 2009). 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 percentage of the lesioned side over the corresponding nonlesioned side. Animals were treated with the 1:1 combination of $\Delta^9$-THC- and CBD-enriched botanical extracts used in the previous experiments, administered 30 min before and 2 hr after the intrastriatal injection of malonate. Malonate-lesioned rats given vehicle, as well as sham-operated animals, were also included in this experiment. All animals were killed 48 hr 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 to be used for qRT-PCR analysis. In all experiments, five or six animals were used per experimental group.

### HPLC Analysis of GABA Concentrations

Brain coronal slices (approximately 500 μm thick) were made at levels containing the caudate-putamen, according to Palkovits and Brownstein (1988). Subsequently, this structure was dissected and homogenized in 20–40 volumes of cold 150 mM potassium phosphate buffer, pH 6.8. Homogenates were diluted (1:2) with 0.4 N perchloric acid containing 0.4 mM sodium disulfite, 0.90 mM EDTA, and β-aminobutiric acid as internal standard. Afterward, samples were centrifuged for 3 min (15,000*g*) and the supernatants subjected to a derivatization process with o-phthaldehide (OPA)-sulfite solution (14.9 mM OPA, 45.4 mM sodium sulfite, and 4.5% ethanol in 327 mM borate buffer, pH 10.4) prior to their injection into the HPLC system. This system consisted of an isocratic Spectra-Physics 8810 pump and on an RP-18 column (Spherisorb ODS-2; 150 mm, 4.6 mm, 5 μm particle size; Waters). The mobile phase, previously filtered and degassed, consisted of 0.06 M sodium dihydrogen phosphate, 0.06 mM EDTA, and 20–30% methanol (pH 4.4). The flow rate was 0.8 ml/min. The effluent was monitored with a Metrohm bioanalytical system amperometric detector using a glassy carbon electrode. The potential was 0.85 V relative to an Ag/AgCl reference electrode with a sensitivity of 50 nA (approximately 2 ng per sample). The signal was recorded on a Spectra-Physics 4290 integrator. The results were obtained from the peaks and calculated by comparison with the area under the corresponding internal standard peak. Values were expressed as micrograms per total tissue analyzed.

### Histological Analyses

Coronal brain sections (20 μm thick) were obtained with a cryostat and collected on gelatin-coated slides. Slices obtained at the level of the caudate-putamen were used for Nissl staining (see details in Alvarez et al., 2008), which per-

mitted determination of the effects of particular treatments on cell number and for immunohistochemical analysis of OX-42, a marker of reactive microglia, and GFAP, a marker of astrocytes. For immunohistochemistry, sections were incubated overnight at 30°C with 1) monoclonal anti-rat CD11b antibody (AbD Serotec, Oxford, United Kingdom) used at 1:500 or 2) monoclonal anti-rat GFAP (Sigma-Aldrich, Madrid, Spain) used at 1:500. After incubation with the corresponding primary antibody, sections were washed in 0.1 M PBS and incubated for 2 hr at 37°C with the appropriate mouse, rat, and rabbit highly cross-adsorbed AlexaFluor 546 (for GFAP) or 488 (for OX-42) secondary antibody (Invitrogen, Carlsbad, CA) used at 1:400. 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 performed in ImageJ, the software developed and freely distributed by the U.S. National Institutes of Health.

### Real-Time qRT-PCR Analysis

Total RNA was isolated from striata using RNATidy reagent (AppliChem, Cheshire, CT). The total amount of RNA extracted was quantitated by spectrometry at 260 nm and its purity from the ratio of the absorbance values at 260 and 280 nm, whereas its integrity was confirmed in agarose gels. After genomic DNA had been removed (to eliminate DNA contamination), single-stranded complementary DNA was synthesized from 1 μg total RNA using a commercial kit (Rneasy Mini Quantitect Reverse Transcription; Qiazen, Izasa, Madrid, Spain). The reaction mixture was kept frozen at –20°C until enzymatic amplification. Quantitative real-time PCR assays were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) to quantify mRNA levels for IGF-1 (ref. Rn99999087_m1), $CB_1$ receptor (ref. Rn00562880_m1), calpain (ref. Rn00569689_m1), SOD-1 (ref. Rn00566938_m1), and iNOS (ref. Rn00561646_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), and the threshold cycle was calculated by the instrument's software (7300 Fast System).

### Statistical Analysis

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

## RESULTS

### Effects of Phytocannabinoids on 3NP-Induced Striatal Damage

As expected, the administration of 3NP damaged the striatum as reflected by GABA deficiency ($F_{2,17} = 3.79$, $P < 0.05$; Fig. 1), a reduction in the number of Nissl-stained neurons ($F_{2,11} = 5.94$, $P < 0.05$; Fig. 1), and a lowered expression of the antioxidant enzyme SOD-1 ($F_{2,14} = 3.82$, $P < 0.05$; Fig. 2) and the neurotrophic factor IGF-1 ($F_{2,16} = 3.73$, $P < 0.05$; Fig. 2). These changes, although found in a specific experimental model of HD, have also been found in HD patients using CSF, blood cell, and postmortem brain samples (Manyam et al., 1981; Storey and Beal, 1993; Boll et al., 2008). They include changes in SOD-1 expression and also in GABA concentration, despite the fact that the reduction in this neurotransmitter found here after 3NP intoxication was very modest (Fig. 1). This also happens in patients and reflects the selectivity in the degeneration of GABA-containing neurons in both patients and 3NP-lesioned rats that includes striatal projection neurons but not other GABA-containing neurons such as striatal interneurons (for review see Pazos et al., 2008). 3NP intoxication also caused an up-regulation of the calcium-binding protein calpain ($F_{2,15} = 3.92$, $P < 0.05$; Fig. 2), as reported previously (Bizat et al., 2003a,b; Brouillet et al., 2005), and it dramatically impaired the expression of $CB_1$ receptors in the striatum ($F_{2,13} = 4.12$, $P < 0.05$; Fig. 2), a change that has also been detected in patients (for review see Pazos et al., 2008) and transgenic mouse models of HD, even at presymptomatic phases (Blázquez et al., 2011). Although key cytotoxic events after 3NP intoxication are related to mitochondrial dysfunction, oxidative stress, and calpain activation, we also analyzed a few parameters related to glial activation in these animals and found elevated immunostaining for OX-42, a marker of reactive microgliosis, that was restricted to a small area in the striatal parenchyma (Fig. 1). However, other proinflammatory markers analyzed such as iNOS or GFAP, a marker of astrogliosis, were not affected after 3NP intoxication, in accordance with the idea that inflammatory events are modest in this HD model (Bantubungi et al., 2005). All these responses are compatible with 3NP selectively damaging the striatum even though this neurotoxin was systemically administered (for review see Brouillet et al., 2005). As mentioned above, this selective damage is consistent with some of the pathogenic changes that occur in HD patients, particularly those that relate to oxidative injury and calpain activation.

The administration of a mixture of $\Delta^9$-THC- and CBD-enriched botanical extracts (1:1, as in Sativex) attenuated to different extents all these 3NP-induced changes. For example, it slightly reduced GABA depletion (Fig. 1), as indicated by the loss of statistical significance of 3NP-lesioned rats vs. controls after administration of cannabinoids. The same type of response (loss of statistical significance of the effect found in 3NP-lesioned rats vs. controls) was found for the effect of $\Delta^9$-THC- and CBD-enriched botanical extracts on 3NP-induced loss of Nissl-stained neurons (Fig. 1), down-regulation of the $CB_1$ receptor and of IGF-1 expression (Fig. 2), and up-regulation of calpain (Fig. 2). However, this mixture completely reversed the reduction in SOD-1 expression; the differences between vehicle- and cannabinoid-injected 3NP-lesioned rats reached statistically significant differences in this case

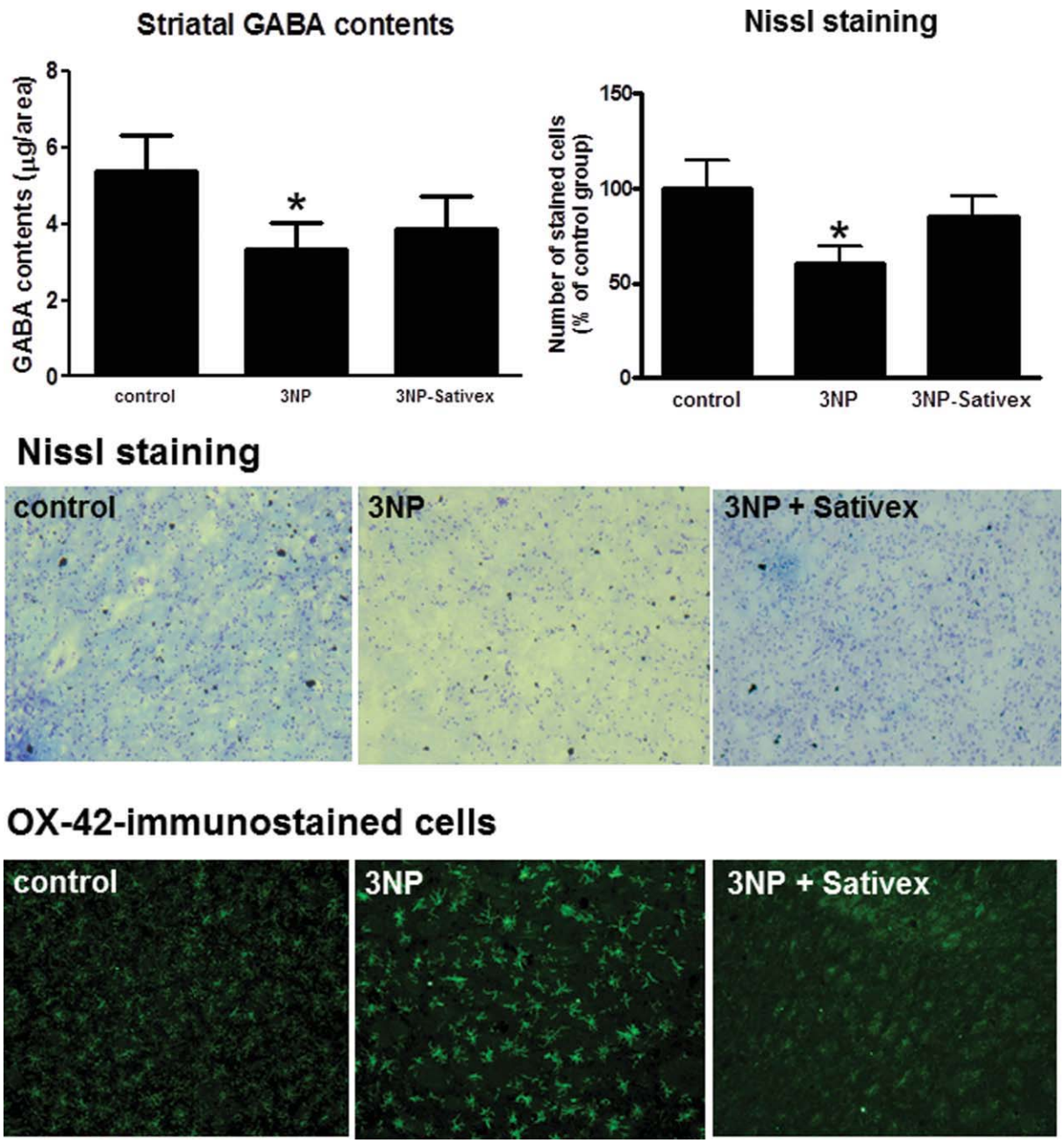


Fig. 1. GABA content, number of Nissl-stained neurons, and OX-42 immunoreactivity measured in the caudate-putamen of rats subjected to systemic injections of 3-nitropropionic acid (3NP) for 5 days and receiving a daily injection of $\Delta^9$-THC- and CBD-enriched botanical extracts combined in a Sativex-like ratio 1:1 (total cannabinoid dose equivalent to 3 mg/kg weight) or vehicle (Tween 80-saline). For details see text. Values are expressed as means ± SEM for five or six animals per group (three or four per group in the case of histological analyses). The Nissl staining data correspond to percentage over the control group. Data were subjected to one-way analysis of variance followed by the Student-Newman-Keuls test (*$P < 0.05$ compared with controls). Representative Niss-stained and OX-42-immunostained microphotographs for each experimental group are included. 20×. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Fig. 2). In addition, the administration of a mixture of $\Delta^9$-THC- and CBD-enriched botanical extracts also attenuated the enhanced OX-42 immunostaining (Fig. 1). These findings support the notion that the combination of both phytocannabinoid botanical extracts may be effective as a neuroprotective therapy, as has been found previously when they are administered alone as pure compounds (Lastres-Becker et al., 2004;

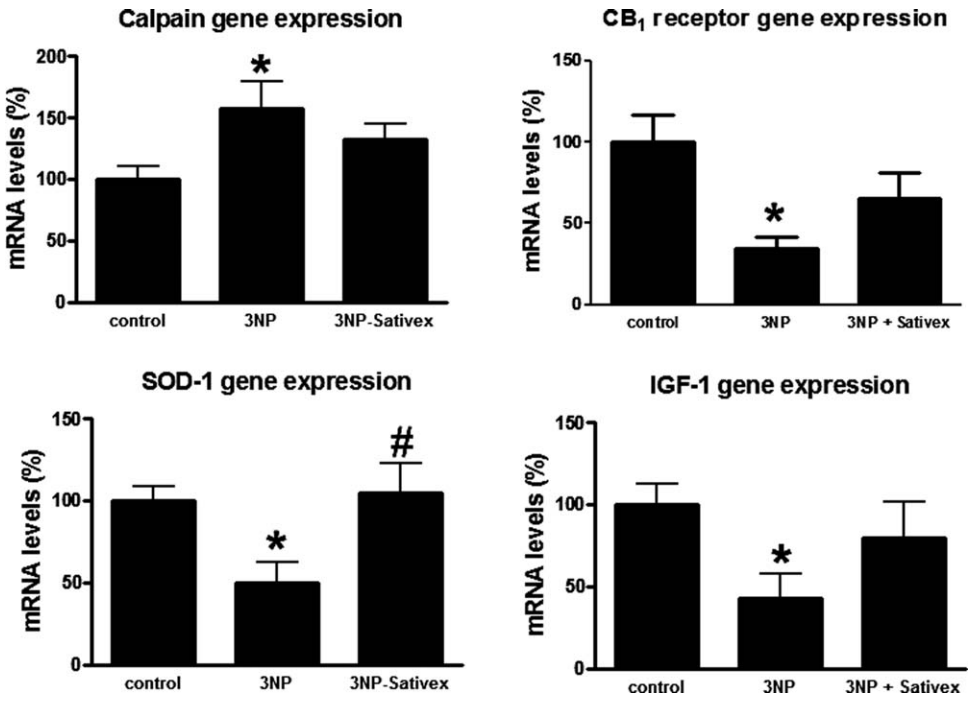


Fig. 2. Calpain, $CB_1$ receptor, SOD-1, and IGF-1 gene expression measured in the caudate-putamen of rats subjected to systemic injections of 3-nitropropionic acid (3NP) for 5 days and receiving a daily injection of $\Delta^9$-THC- and CBD-enriched botanical extracts combined in a Sativex-like ratio 1:1 (total cannabinoid dose equivalent to 3 mg/kg weight) or vehicle (Tween 80-saline). For details see text. Values correspond to percentage over the control group and are expressed as means ± SEM for five or six 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 3NP + vehicle).

Sagredo et al., 2007). In this study, we also performed experiments with other combinations of $\Delta^9$-THC- and CBD-enriched botanical extracts, i.e., 2:1 and 1:2, but always maintaining the same total amount of cannabinoid administered. The objective of this additional testing was to elucidate whether increasing $CB_1$ and $CB_2$ receptor-mediated effects (in the case of the 2:1 combination of $\Delta^9$-THC and CBD) or augmenting cannabinoid receptor-independent effects of phytocannabinoids (in the case of the 1:2 combination of $\Delta^9$-THC and CBD) would enhance or diminish the effects of the Sativex-like 1:1 combination. Our data revealed that changes in SOD-1 expression elicited by 3NP ($F_{4,23} = 4.01$, $P < 0.05$) were totally reversed after the treatment with phytocannabinoids and to a similar extent with the three combinations used in this study (Fig. 3). In addition, the treatment with phytocannabinoids reversed the changes elicited by 3NP in calpain expression ($F_{4,24} = 3.91$, $P < 0.05$), also to a similar extent with the three combinations (Fig. 3), although in this case the reversal was smaller (evident only by the loss of statistical significance for the effect found in 3NP-lesioned rats vs. controls).

## Effects of $CB_1$ and $CB_2$ Receptor Antagonists on the Beneficial Effects of Phytocannabinoids on 3NP-Induced Striatal Damage

The finding that the three combinations of $\Delta^9$-THC- and CBD-enriched botanical extracts induced equivalent degrees of neuroprotection against 3NP intoxication strongly favors the idea that these effects are due to the antioxidant and cannabinoid receptor-independent properties of both phytocannabinoids rather than to their activation of $CB_1$ and/or $CB_2$ receptors. We wanted to see whether we could confirm this notion by conducting additional experiments with selective antagonists for $CB_1$ and $CB_2$ receptor types, i.e., SR141716 and AM630, respectively. Although 3NP again up-regulated calpain expression, and phytocannabinoids (combined $\Delta^9$-THC- and CBD-enriched botanical extracts, 1:1) prevented these responses ($F_{4,24} = 57.28$, $P < 0.005$; see Fig. 4; note that, in this experiment, the effect of phytocannabinoids was more pronounced than in the previous experiments insofar as the differences between vehicle- and phytocannabinoid-injected 3NP-lesioned rats did reach statistical significance), neither SR141716 nor AM630 antagonized the protective effects of these phytocannabinoids (Fig. 4). In addition, the administration of SR141716 or AM630 in the absence of phytocannabinoid extracts did not alter 3NP-induced up-regulation of calpain (data not shown).

## Effects of Phytocannabinoids on Malonate-Induced Striatal Damage

We also wanted to determine whether a Sativex-like combination of $\Delta^9$-THC- and CBD-enriched botanical extracts might also protect striatal neurons in a different ex-

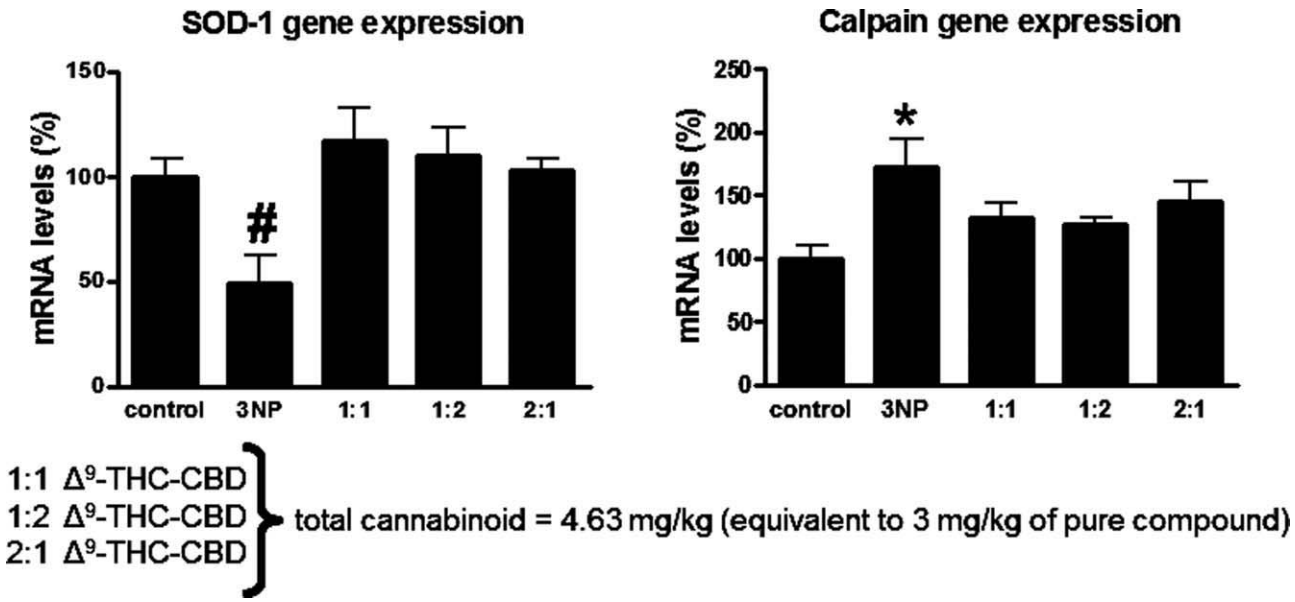


Fig. 3. SOD-1 and calpain gene expression measured in the caudate-putamen of rats subjected to systemic injections of 3-nitropropionic acid (3NP) for 5 days and receiving a daily injection of one of three different combinations of $\Delta^9$-THC- and CBD-enriched botanical extracts, 1:1 (as in Sativex), 1:2, and 2:1 (total cannabinoid dose equivalent to 3 mg/kg weight for each of the three combinations), or vehicle (Tween 80-saline). For details see text. Values correspond to percentage over the control group and are expressed as means ± SEM for five or six animals per group. Data were subjected to one-way analysis of variance followed by the Student-Newman-Keuls test ($^*P < 0.05$ compared only with controls; $^{\#}P < 0.05$ compared with controls and also the other groups).

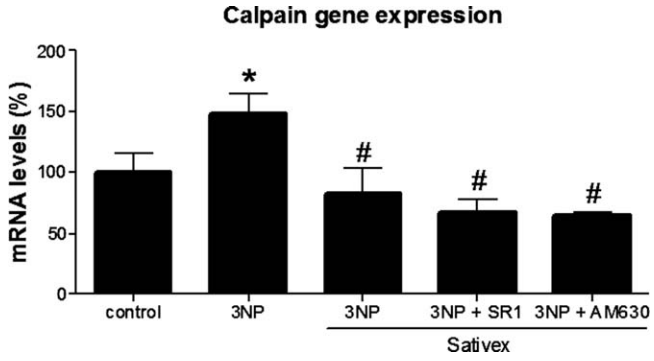


Fig. 4. Calpain gene expression measured in the caudate-putamen of rats subjected to systemic injections of 3-nitropropionic acid (3NP) for 5 days and receiving a daily administration of $\Delta^9$-THC- and CBD-enriched botanical extracts combined in a Sativex-like ratio 1:1 (total cannabinoid dose equivalent to 3 mg/kg weight), selective antagonists for the $CB_1$ (SR141716) or the $CB_2$ (AM630) receptor, or vehicle (Tween 80-saline). For details see text. Values correspond to percentage over the control group and are expressed as means ± SEM for five or six 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 3NP).

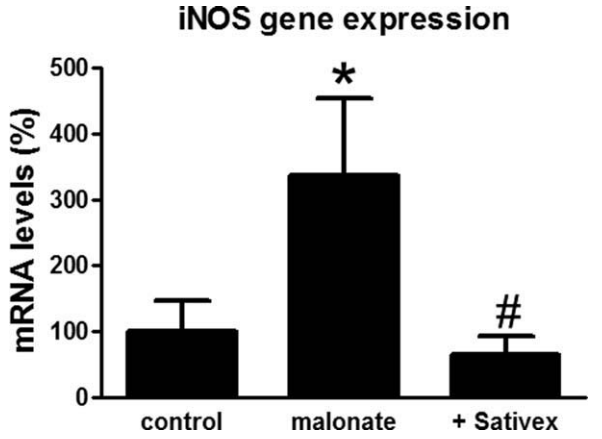


Fig. 5. iNOS gene expression measured in the caudate-putamen of rats subjected to unilateral intrastriatal administration of malonate and receiving two injections of $\Delta^9$-THC- and CBD-enriched botanical extracts combined in a Sativex-like ratio 1:1 (total cannabinoid dose equivalent to 3 mg/kg weight) or vehicle (Tween 80-saline). For details see text. Values correspond to percentage over the control group and are expressed as means ± SEM for five or six 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).

perimental model of HD. We used malonate-lesioned rats in which striatal damage is frequently accompanied by up-regulatory responses of different inflammatory markers (i.e., COX-2, iNOS, tumor necrosis factor-α; for details see Sagredo et al., 2009). In the present study, we focused on iNOS, because this enzyme is strongly up-regulated by malonate injection (Sagredo et al., 2009), a fact also observed in our experiments ($F_{2,11} = 5.783$, $P < 0.05$; Fig. 5). Interestingly, the treatment with a Sativex-like combination of $\Delta^9$-THC- and CBD-enriched botanical

extracts reduced the malonate-induced up-regulation of iNOS expression to levels similar to controls (Fig. 5).

## DISCUSSION

As mentioned in the introductory paragraphs, chronic administration of specific cannabinoids has proven neuroprotective effects in HD (Pazos et al., 2008), which adds to their ability to contribute to the suppression of certain motor symptoms (e.g., hyperkinetic movements) because of their hypokinetic profile (Lastres-Becker et al., 2002, 2003). The evidence for this is already strong; signs of such neuroprotective effects have been observed in a variety of experimental models of HD (e.g., generated by mitochondrial or excitotoxic neurotoxins, transgenic mice, etc.) and produced by various types of cannabinoid compounds ($CB_1$ or $CB_2$ agonists, antioxidant cannabinoids, etc.; for recent review see Pazos et al., 2008; Fernández-Ruiz et al., 2010). Therefore, there is now enough evidence for clinically evaluating a cannabis-based medicine in HD patients. In fact, previous studies have already tried to determine whether specific cannabinoids are efficacious in HD, although all have concentrated on HD symptoms rather than on disease progression. For example, nabilone, a $\Delta^9$-THC analog that activates $CB_1$ receptors, was assayed in two uncontrolled, single-patient studies. However, these studies yielded conflicting results. Thus, although nabilone induced signs of improvement in one of these studies (Curtis and Rickards, 2006), in the other study it made symptoms worse (Müller-Vahl et al., 1999). More recently, nabilone was also used in a double-blind, placebo-controlled, crossover study (Curtis et al., 2009) in which it induced improvements in various motor and cognitive indices. Finally, CBD has also been studied in a controlled trial, showing no effect on chorea severity in 15 HD patients (Consroe et al., 1991).

It is also important to note that all these previous trials did not examine disease progression and were conducted with individual cannabinoid agonists, whereas the data obtained in animal models more recently suggest, as mentioned above, that combinations of different cannabinoids or the use of a broad-spectrum cannabinoid is to be recommended for clinical testing of neuroprotective effects given the diversity of targets and cytotoxic processes in which cannabinoid compounds may afford positive results. This might explain the lack of positive effects found in some of these previous trials. In this context, we believe that a good choice may be the recently licenced cannabis-based medicine Sativex. This medicinal preparation is an equimolecular combination of $\Delta^9$-THC- and CBD-enriched botanical extracts (for recent review see Russo and Guy, 2006; Wright, 2007; Pertwee, 2009). Both $\Delta^9$-THC and CBD have already been investigated in animal models of HD (Lastres-Becker et al., 2004; Sagredo et al., 2007). However, since the two were administered by themselves but not in combination, it was of interest, before testing Sativex in the clinic, to evaluate a Sativex-like mixture of $\Delta^9$-THC and CBD in the same animal model in which both $\Delta^9$-THC and CBD alone have been found to induce neuroprotection. This was the objective of the present study. Collectively, our data demonstrate that a Sativex-like 1:1 combination of $\Delta^9$-THC- and CBD-enriched botanical extracts protected striatal neurons against 3NP toxicity. We found that the different neurochemical (GABA depletion), molecular (up-regulation of calpain and losses of IGF-1, $CB_1$ receptors, and SOD-1), and histological (loss of Nissl-stained cells and increased OX-42 immunoreactivity) alterations associated with HD pathogenesis and efficaciously reproduced in the experimental model of 3NP-induced striatal degeneration (see key references in the introductory paragraphs) were totally or partially attenuated by treatment with the Sativex-like 1:1 combination of $\Delta^9$-THC and CBD. Some of these effects had already been observed to be produced by CBD or $\Delta^9$-THC administered alone as pure compounds in the same HD model, 3NP-lesioned rats (Lastres-Becker et al., 2004; Sagredo et al., 2007). In addition, these or similar effects have also been seen when these two compounds or other cannabinoids were evaluated as neuroprotective agents in other models of HD (Pintor et al., 2006; Sagredo et al., 2009; Palazuelos et al., 2009; Blázquez et al., 2011). In fact, we also wanted to examine here whether the Sativex-like combination of $\Delta^9$-THC- and CBD-enriched botanical extracts may afford neuroprotection in a HD model other than 3NP-lesioned animals. We used malonate-lesioned rats, in which striatal damage is frequently associated with up-regulation of proinflammatory factors, and we found that Sativex-like combination was also effective in this model. Therefore, all the evidence collected here and coming from previous studies strongly supports the hypothesis that Sativex is an effective medicine for the treatment of HD patients, prompting an urgent need for its clinical evaluation.

Additional support for conducting such a clinical evaluation comes from studies conducted here with other combinations of $\Delta^9$-THC- and CBD-enriched botanical extracts in 3NP-lesioned rats. In addition to the 1:1 combination of $\Delta^9$-THC and CBD, two other combinations were studied with the objective of seeing whether the beneficial effects of the Sativex-like combination could be improved by increasing $CB_1$ and $CB_2$ receptor-mediated effects (in the case of the combination 2:1 of $\Delta^9$-THC and CBD) or by potentiating CBD effects that are $CB_1$ and $CB_2$ receptor independent (in the case of the combination 1:2 of $\Delta^9$-THC and CBD). In both cases, we reached levels of neuroprotection that were similar to those produced by the Sativex-like 1:1 combination of $\Delta^9$-THC and CBD, which would support the idea that these neuroprotective effects are mediated by cannabinoid receptor-independent mechanisms. This was confirmed in additional experiments aimed at determining whether coadministration of the 1:1 combination of $\Delta^9$-THC and CBD with either SR141716 (a selective $CB_1$ receptor antagonist) or AM630 (a selective $CB_2$ receptor blocker) attenuated the effects of this cannabinoid mixture. Neither SR141716 nor AM630 reduced its effects, supporting the

idea that the effects were not mediated by $CB_1$ or $CB_2$ receptors, in accordance with similar experiments conducted with CBD administered alone and as a pure compound (Sagredo et al., 2007).

Therefore, in absence of additional experimentation, we attribute the protective effects of $\Delta^9$-THC and CBD in 3NP-lesioned rats to their antioxidant properties, which are of similar magnitude for both phytocannabinoids (Hampson et al., 1998; Marsicano et al., 2002; Lastres-Becker et al., 2005) and comparable, or even superior, to those reported for classic antioxidants such as ascorbate or α-tocopherol (Hampson et al., 2000). Both cannabinoids may act as scavengers of reactive oxygen species that are produced by mitochondial defects after 3NP exposure in laboratory animals (Fontaine et al., 2000), replicating the situation in HD patients, which is also associated with an increased generation of reactive oxygen species (Tabrizi et al., 1999). In this sense, the antioxidant action of phytocannabinoids might then be equivalent to the neuroprotective action of other known antioxidant compounds, such as N-acetylcysteine, S-allylcysteine, coenzyme Q10, taurine, the flavonoid kaempferol, vitamins C and E, ginseng components, melatonin, or dehydroepiandrosterone, all of which are highly effective at protecting the brain against 3NP-induced neurotoxicity or in similar HD models (Fontaine et al., 2000; Tadros et al., 2005; Túnez et al., 2005; Nam et al., 2005; Herrera-Mundo et al., 2006; Lagoa et al., 2009; Yang et al., 2009; Kalonia et al., 2010). It is possible, however, that this antioxidant/neuroprotective effect of phytocannabinoids involves the activation of signaling pathways implicated in the control of redox balance (e.g., nrf-2/ARE), as suggested recently for cystamine (Calkins et al., 2010). It is well known that nrf-2 activation is neuroprotective against a variety of cytotoxic stimuli, including 3NP (Calkins et al., 2005), and indeed such activation may constitute a common mechanism of action for a range of different antioxidants, including phytocannabinoids. If this is so, it could be that there is a cannabinoid receptor/target, other than $CB_1$ or $CB_2$ receptors, that can be coupled to the activation of nrf-2 signaling. We are presently exploring this possibility.

In summary, the present study demonstrates that combinations of $\Delta^9$-THC and CBD botanical extracts reduce the striatal atrophy generated by 3NP intoxication. This capability seems to be based mainly on the antioxidant and cannabinoid receptor-independent actions of these phytocannabinoids. In addition, a Sativex-like combination of $\Delta^9$-THC and CBD botanical extracts was also effective in an alternative model of striatal damage in which inflammatory events are more relevant. In our opinion, these data provide sufficient preclinical evidence to justify a clinical evaluation of the cannabis-based medicine Sativex as a neuroprotective agent capable of delaying disease progression in patients affected by HD, a disorder that is currently poorly managed in the clinic, prompting an urgent need for clinical trials with agents showing positive results in preclinical studies.

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