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RESEARCH PAPER

# Symptom-relieving and neuroprotective effects of the phytocannabinoid $\Delta^9$-THCV in animal models of Parkinson's disease

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## BACKGROUND AND PURPOSE
Previous findings have indicated that a cannabinoid, such as $\Delta^9$-THCV, which has antioxidant properties and the ability to activate $CB_2$ receptors but to block $CB_1$, might be a promising therapy for alleviating symptoms and delaying neurodegeneration in Parkinson's disease (PD).

## EXPERIMENTAL APPROACH
The ability of $\Delta^9$-THCV to reduce motor inhibition and provide neuroprotection was investigated in rats lesioned with 6-hydroxydopamine and in mice lesioned with lipopolysaccharide (LPS).

## KEY RESULTS
Acute administration of $\Delta^9$-THCV attenuated the motor inhibition caused by 6-hydroxydopamine, presumably through changes in glutamatergic transmission. Moreover, chronic administration of $\Delta^9$-THCV attenuated the loss of tyrosine hydroxylase–positive neurones caused by 6-hydroxydopamine in the substantia nigra, through an effect related to its antioxidant properties (it was reproduced by cannabidiol -enriched botanical extract). In addition, $CB_2$ receptor–deficient mice responded to 6-hydroxydopamine in a similar manner to wild-type animals, and $CB_2$ receptors were poorly up-regulated in the rat substantia nigra in response to 6-hydroxydopamine. By contrast, the substantia nigra of mice that had been injected with LPS exhibited a greater up-regulation of $CB_2$ receptors. In these animals, $\Delta^9$-THCV also caused preservation of tyrosine hydroxylase–positive neurones. This effect probably involved $CB_2$ receptors as it was also elicited by the selective $CB_2$ receptor agonist, HU-308, and $CB_2$ receptor–deficient mice were more vulnerable to LPS lesions.

## CONCLUSIONS AND IMPLICATIONS
Given its antioxidant properties and its ability to activate $CB_2$ but to block $CB_1$ receptors, $\Delta^9$-THCV has a promising pharmacological profile for delaying disease progression in PD and also for ameliorating parkinsonian symptoms.

## LINKED ARTICLES
This article is part of a themed issue on Cannabinoids in Biology and Medicine. To view the other articles in this issue visit http://dx.doi.org/10.1111/bph.2011.163.issue-7

## Abbreviations
CBD, cannabidiol; CP-55940, 2-[(1*R*,2*R*,5*R*)-5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-5-(2-methyloctan-2-yl)phenol; HU-308, [(1*R*,2*R*,5*R*)-2-[2,6-dimethoxy-4-(2-methyloctan-2-yl)phenyl]-7,7-dimethyl-4-bicyclo[3.1.1]hept-3-enyl] methanol; PD, Parkinson's disease; $\Delta^9$-THC, $\Delta^9$-tetrahydrocannabinol; $\Delta^9$-THCV, $\Delta^9$-tetrahydrocannabivarin

## Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder; its aetiology is associated with environmental insults, genetic susceptibility or interactions between both causes (Thomas and Beal, 2007). The major clinical symptoms in PD are tremor, bradykinesia, postural instability and rigidity, symptoms that result from the severe dopaminergic denervation of the striatum caused by the progressive death of dopaminergic neurones of the substantia nigra pars compacta (Nagatsu and Sawada, 2007). Major symptoms in PD (e.g. bradykinesia) can be attenuated with dopaminergic replacement therapy (Thomas and Beal, 2007). However, this therapy does not work for all PD patients, and when used for more than 5–10 years, it provokes an irreversible dyskinetic state (Pezzoli and Zini, 2010). Therefore, the search for novel symptomatic therapies, as well as for treatments effective in delaying the progression of nigrostriatal damage in PD, is still the major challenge in PD therapy.

Cannabinoid-based compounds have recently been proposed as promising therapies in PD (García-Arencibia *et al.*, 2009). Thus, the blockade of $CB_1$ receptors, which are highly abundant in basal ganglia structures, may be effective in reducing the motor inhibition typical of PD patients (Fernández-Espejo *et al.*, 2005; González *et al.*, 2006; Kelsey *et al.*, 2009), as well as in enhancing the therapeutic effect of moderate doses of levodopa, which are less pro-dyskinetic (Kelsey *et al.*, 2009). Both effects are in accordance with the overactivity of the cannabinoid system observed in PD patients and in animal models of this disease (Di Marzo *et al.*, 2000; Romero *et al.*, 2000; Lastres-Becker *et al.*, 2001; Gubellini *et al.*, 2002; Pisani *et al.*, 2005). In addition, $CB_1$ receptor–deficient mice also showed less severe dyskinesias, when lesioned with 6-hydroxydopamine and treated with levodopa, compared with wild-type animals (Pérez-Rial *et al.*, 2010). However, the efficacy of $CB_1$ receptor blockade was restricted to specific circumstances, that is the use of low doses (Fernández-Espejo *et al.*, 2005; González *et al.*, 2006; Kelsey *et al.*, 2009) and very extensive nigral damage (Fernández-Espejo *et al.*, 2005), conditions that were not reproduced in the only clinical trial conducted so far with $CB_1$ receptor blockers, which included a population of patients that were all good responders to levodopa (Mesnage *et al.*, 2004). Therefore, this potential therapeutic strategy merits further clinical investigation, this time with PD patients that respond poorly to levodopa. On the other hand, other studies have reported that compounds that directly or indirectly activate rather than block $CB_1$ receptors also alleviate the symptoms associated with PD (Ferrer *et al.*, 2003; Segovia *et al.*, 2003; Fernández-Espejo *et al.*, 2004), thus stressing the complexity of the effects of cannabinoids in PD.

Some cannabinoids have been reported to protect nigral neurones from death caused by different cytotoxic stimuli in various experimental models of PD (Lastres-Becker *et al.*, 2005; García-Arencibia *et al.*, 2007; Jiménez-Del-Rio *et al.*, 2008). These include the phytocannabinoids, $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) and cannabidiol (CBD) (Lastres-Becker *et al.*, 2005; García-Arencibia *et al.*, 2007), the synthetic $CB_1/CB_2$ receptor agonist CP55 940 (Jiménez-Del-Rio *et al.*, 2008) and the anandamide analogue AM404 (García-Arencibia *et al.*, 2007). *A priori* these compounds acted through antioxidant mechanisms that seem to be independent of $CB_1$ or $CB_2$ receptors, although selective $CB_2$ receptor agonists also showed efficacy in MPTP-lesioned mice (Price *et al.*, 2009) but not in 6-hydroxydopamine-lesioned rats (García-Arencibia *et al.*, 2007). It is also noteworthy that $CB_1$ receptor–deficient mice display an increased vulnerability to 6-hydroxydopamine lesions (Pérez-Rial *et al.*, 2010). However, selective $CB_1$ receptor agonists do not seem to protect against 6-hydroxydopamine-induced damage (García-Arencibia *et al.*, 2007), and they may even aggravate major parkinsonian symptoms, given the hypokinetic effects associated with the activation of the $CB_1$ receptor (García-Arencibia *et al.*, 2009).

Therefore, these previous data provide good evidence that a cannabinoid having antioxidant properties and the ability to activate $CB_2$ receptors but to block $CB_1$ receptors, might serve to alleviate parkinsonian symptoms and to arrest/delay neurodegeneration in PD. $\Delta^9$-THC and CBD are two phytocannabinoids that display cannabinoid receptor–independent antioxidant properties in PD (Lastres-Becker *et al.*, 2005; García-Arencibia *et al.*, 2007). However, $\Delta^9$-THC is an agonist of $CB_1$ receptors and acutely aggravates motor inhibition, and CBD has negligible activity at both $CB_1$ and $CB_2$ receptors. In addition, although rimonabant may be efficacious as a symptom-relieving agent (Fernández-Espejo *et al.*, 2005; González *et al.*, 2006; Kelsey *et al.*, 2009), it does not appear to have neuroprotective effects in PD. Indeed, enhanced damage has been reported after genetic ablation of $CB_1$ receptors and lesion with 6-hydroxydopamine (Pérez-Rial *et al.*, 2010). However, the phytocannabinoid $\Delta^9$-tetrahydrocannabivarin ($\Delta^9$-THCV) has a pharmacological profile that seems to be particularly appropriate for PD; it is an antioxidant and appears to act as a $CB_1$ receptor antagonist (when used at doses lower than 3 $mg \cdot kg^{-1}$) but is a potent $CB_2$ receptor agonist. *In vitro*, it has been shown to displace [$^3$H]-CP55940 from human $CB_1$ and $CB_2$ receptors, antagonize CP55940-induced stimulation of [$^{35}$S]-GTP$\gamma$S binding to human $CB_1$ receptors, inhibit forskolin-induced stimulation of cAMP production in human $CB_2$-expressing Chinese hamster ovary cells and stimulate [$^{35}$S]-GTP$\gamma$S binding both to human $CB_2$ receptors and to mouse spleen membranes, all at submicromolar concentrations (Thomas *et al.*, 2005; Pertwee *et al.*, 2007; Bolognini *et al.*, 2010). *In vivo*, it has been shown to antagonize certain $CB_1$ receptor–mediated behavioural effects of $\Delta^9$-THC in mice (Pertwee *et al.*, 2007) and decrease carrageenan-induced mouse paw oedema in a manner that can be antagonized by the $CB_2$ antagonist, SR144528, but not by the $CB_1$ antagonist, rimonabant (Bolognini *et al.*, 2010).

The aim of the present study was to demonstrate that $\Delta^9$-THCV can alleviate the symptoms associated with PD by blocking $CB_1$ receptors at low doses (Thomas *et al.*, 2005) and also induce neuroprotection due to its antioxidant properties and/or $CB_2$ agonist activity. We used rats subjected to i.c.v. injection of 6-hydroxydopamine as a model to screen for evidence of amelioration and neuroprotection (Rodriguez Diaz *et al.*, 2001; see details in González *et al.*, 2006; García-Arencibia *et al.*, 2008). In an initial experiment, we compared the ability of $\Delta^9$-THCV to improve the motor inhibition produced by 6-hydroxydopamine in these animals with that of rimonabant, a compound that has already been shown to have beneficial effects in this experimental model (González

*et al.*, 2006). Concentrations of neurotransmitters in the basal ganglia of these animals were determined, in particular those of glutamate as this neurotransmitter has been found to be involved in the anti-parkinsonian effect that follows the blockade of $CB_1$ receptors (García-Arencibia *et al.*, 2008). In a second experiment, $\Delta^9$-THCV was administered to 6-hydroxydopamine-lesioned animals for a period of 14 days and its ability to protect nigral neurones from 6-hydroxydopamine insult was evaluated by tyrosine hydroxylase immunostaining, a parameter that, although does not completely reflect neurodegeneration/neuroprotection *per se*, is frequently used for this purpose. OX-42 immunostaining (microglial activation) was also determined. Given that $CB_2$ receptor agonists failed to provide neuroprotection in 6-hydroxydopamine-lesioned rats (García-Arencibia *et al.*, 2007), effects of $\Delta^9$-THCV in these rats should be assigned *a priori* only to its cannabinoid receptor–independent antioxidant properties. We explored this hypothesis further: (i) by comparing the effects of $\Delta^9$-THCV with those produced by CBD, which showed positive effects in previous studies (Lastres-Becker *et al.*, 2005; García-Arencibia *et al.*, 2007); (ii) by determining the presence of $CB_2$ receptors in the substantia nigra of 6-hydroxydopamine-lesioned rats; and (iii) by measuring the response to this neurotoxin in $CB_2$ receptor–deficient mice. As our data confirmed that the neuroprotective effects of $\Delta^9$-THCV in 6-hydroxydopamine-lesioned rats do not appear to be mediated by $CB_2$ receptors, we conducted additional experiments using mice intrastriatally injected with LPS, a model of PD with a higher inflammatory profile and in which up-regulation of $CB_2$ receptors in response to damage was much more intense. HU-308, a selective $CB_2$ receptor agonist, was used as a positive control. The response of $CB_2$ receptor–deficient mice to LPS was also investigated.

## Methods

### *6-Hydroxydopamine or LPS lesions and pharmacological treatments*

*Animals.* Male Sprague–Dawley rats or $CB_2^{-/-}$ mice and their respective wild-type littermates (bred in our animal facilities from mice donated by Dr Nancy Buckley, California State Polytechnic University, Pomona, CA, USA; see Buckley *et al.*, 2000; Buckley, 2008) were housed in a room with a controlled photoperiod (0600–1800 h light) and temperature (22 ± 1°C). They had free access to standard food and water and were used at adult age (>2 months old and weighing around 350 g at the onset of experiments, for rats; and 3–4 months old; 25–30 g weight, for mice) for experimental purposes. All experiments were conducted according to Spanish and European guidelines (directive 86/609/EEC). In the case of mice, their genetic profile ($CB_2^{+/+}$, $CB_2^{+/-}$ and $CB_2^{-/-}$) was determined by PCR analysis, as described by Buckley *et al.* (2000), using DNA extracted from a piece of tail taken from each mouse. Only homozygous mice ($CB_2^{+/+}$ or $CB_2^{-/-}$) were used in these experiments.

*Intracerebroventricular or unilateral injection of 6-hydroxydopamine or LPS.* Rats were subjected to an i.c.v. injection of 6-hydroxydopamine following the procedure previously described by Rodriguez Diaz *et al.*, (2001). This model has been validated in our laboratory, and the details regarding the dose–response curves and the time course of the effects of 6-hydroxydopamine, as well as the behavioural characterization of motor deficits, and the histopathological and neurochemical evaluation of the animal brains have been described elsewhere (González *et al.*, 2006). Rats were anaesthetized (ketamine 40 $mg \cdot kg^{-1}$ + xylazine 4 $mg \cdot kg^{-1}$, i.p.) 30 min after pretreatment with desipramine (25 $mg \cdot kg^{-1}$, i.p.), and then 6-hydroxydopamine free base (200 μg in a volume of 5 μL of saline containing 0.05% ascorbate to avoid oxidation) or saline (for control rats) was injected stereotaxically into the third ventricle (coordinates from bregma: –2 mm AP, 0 mm ML and 8 mm DV, according to Paxinos and Watson, 1986). The solution was injected slowly (0.5 $\mu L \cdot 30\ s^{-1}$), and the needle was left in place for 5 min before being slowly withdrawn. This avoids generating reflux and a rapid increase in intracranial pressure. After the application of 6-hydroxydopamine or saline, animals were used for pharmacological treatments as described in the following section. We also induced lesions with 6-hydroxydopamine using unilateral administration, the advantage of which is that contralateral structures serve as controls for the different analyses. This procedure was used in rats to seek out a possible up-regulation of $CB_2$ receptors in the lesioned substantia nigra and in mice to evaluate possible differences in the response to 6-hydroxydopamine between $CB_2$-deficient and wild-type animals. Rats (pretreated with 25 $mg \cdot kg^{-1}$ desimipramine, i.p., 30 min before) received injections of 6-hydroxydopamine free base (8 μg in a volume of 2 μL of saline containing 0.05% ascorbate to avoid oxidation) stereotaxically into the medial forebrain bundle (coordinates from bregma: –2.5 mm AP, –1.8 mm ML and –8.9 mm DV; Paxinos and Watson, 1986). Two weeks post lesion, animals were transcardially perfused with saline followed by fresh 4% paraformaldehyde [in 0.1 M phosphate-buffered saline (PBS)], and their brains were collected and post-fixed overnight at 4°C, cryoprotected with 30% sucrose in PBS and then frozen and stored at –80°C for immunohistochemical analysis of $CB_2$ receptors. In the case of mice (also pretreated with 25 $mg \cdot kg^{-1}$ desimipramine, i.p., 30 min before), 6-hydroxydopamine (2 μL at a concentration of 2 $\mu g \cdot \mu L^{-1}$ saline in 0.2% ascorbate) was injected into the right striatum at a rate of 0.5 $\mu L \cdot min^{-1}$, using the following coordinates: +0.4 mm AP, ±1.8 mm ML and –3.5 mm DV, as described in Alvarez-Fischer *et al.* (2008). These animals were killed by rapid and careful decapitation 1 week after lesioning, and their brains were rapidly removed and frozen in 2-methylbutane cooled in dry ice and stored at –80°C for subsequent immunohistochemical analysis of the substantia nigra. On the other hand, other groups of mice received unilateral injections of *S. Minnesota* LPS (Sigma-Aldrich, Madrid, Spain) into two points of the right striatum following the procedure developed by Hunter *et al.* (2009). We used the following stereotaxic coordinates from bregma: +1.18 mm AP, –1.5 mm ML and –3.5 mm DV, as well as –0.34 mm AP, –2.5 mm ML and –3.2 mm DV (see details in Hunter *et al.*, 2009). At each intrastriatal coordinate, 5 μg of LPS in a volume of 1 μL of saline was injected by using the same procedure as for 6-hydroxydopamine. Control animals were

sham-operated and injected with 1 μL of saline using the same coordinates. This procedure leads to a loss of dopaminergic neurones caused predominantly by LPS-induced inflammatory events. After the application of LPS or saline, animals were subjected to pharmacological treatments as described in the following section, although a separate group remained untreated for 2 weeks and were perfused with paraformaldehyde following the same procedure as described above. Lastly, LPS was also administered to $CB_2$-deficient and wild-type mice to evaluate possible differences in the response to LPS, following the same procedure described above.

*Pharmacological treatments*. Our initial experiment was directed at establishing whether $\Delta^9$-THCV, at a dose of 2 mg·kg$^{-1}$ (based on previous dose–response analysis conducted in our laboratory; data not shown) can behave *in vivo* as a $CB_1$ receptor antagonist in rats since it has previously been validated as a $CB_1$ receptor antagonist *in vivo* only in mice (Pertwee *et al.*, 2007; Bolognini *et al.*, 2010). We investigated its ability to attenuate two effects induced by the cannabinoid agonist, CP-55,940: motor inhibition and antinociception in a model of acute pain, both effects that are thought to be mediated by $CB_1$ receptors. To this end, naïve rats were administered CP55,940 (0.1 mg·kg$^{-1}$, i.p.; purchased from Tocris Bioscience, Biogen Científica, Madrid, Spain) and/or $\Delta^9$-THCV (kindly provided by GW Pharmaceuticals Ltd, Cambridgeshire, UK) and effects on their motor behaviour were determined in a computer-aided actimeter, followed by the hot-plate test to determine their nociceptive sensitivity. In a subsequent experiment, $\Delta^9$-THCV was administered i.p. in a single dose (2 mg·kg$^{-1}$) to 6-hydroxydopamine-lesioned animals 14 days after the lesion. Separate groups of animals received vehicle (Tween 80 : saline, 1:16) or rimonabant (0.1 mg·kg$^{-1}$; kindly provided by Sanofi-Aventis, Montpellier, France), also i.p and at the same dose employed in our previous studies (González *et al.*, 2006; García-Arencibia *et al.*, 2008). Ten minutes later, the behaviours of vehicle-, $\Delta^9$-THCV- or rimonabant-injected 6-hydroxydopamine-lesioned animals and sham-operated controls were assessed in a computer-aided actimeter for a period of 10 min, at the end of which the animals were killed by rapid and careful decapitation. Their brains were rapidly removed and frozen in 2-methylbutane cooled in dry ice and stored at –80°C for analysis of neurotransmitters in basal ganglia. In the next experiment, $\Delta^9$-THCV (2 mg·kg$^{-1}$) was administered i.p. to 6-hydroxydopamine-lesioned animals for a period of 14 days (one injection per day) starting shortly (approximately 16 h) after the lesion. Separate groups of animals received vehicle (Tween 80 : saline, 1:16) or CBD-enriched botanical extract (also provided by GW Pharmaceuticals Ltd, Cambridgeshire, UK) that contains 64.8% CBD, 2.3% $\Delta^9$-THC, 1.1% cannabigerol, 3.0% cannabichromene and 1.5% other phytocannabinoids. It was also administered i.p., at a dose of 4.63 mg·kg$^{-1}$ (equivalent to 3 mg·kg$^{-1}$ of pure CBD, the same dose used in previous studies; Lastres-Becker *et al.*, 2005; García-Arencibia *et al.*, 2007), and was used in this investigation as a positive control. Two hours after the final injection, the 6-hydroxydopamine-lesioned animals injected with vehicle, $\Delta^9$-THCV or CBD-enriched botanical extract and sham-operated controls were killed by rapid and careful decapitation. Their brains were rapidly removed and frozen in 2-methylbutane cooled in dry ice and stored at –80°C for subsequent immunohistochemical analysis of the substantia nigra. Lastly, $\Delta^9$-THCV (2 mg·kg$^{-1}$) or HU-308 (5 mg·kg$^{-1}$; purchased from Tocris Bioscience, Biogen Científica) were administered i.p. to LPS-lesioned mice for a period of 14 days (one injection per day) starting shortly (approximately 16 h) after the lesion. Separate groups of animals received vehicle (Tween 80 : saline, 1:16). Two hours after the final injection, the LPS-lesioned animals injected with vehicle, $\Delta^9$-THCV or HU-308 and sham-operated controls were killed by transcardial perfusion with 4% paraformaldehyde as described above. Their brains were collected, post-fixed, cryoprotected and subsequently used for immunohistochemical analysis of the substantia nigra.

## Behavioural analysis

*Computer-aided actimeter.* Motor activity was analysed in a computer-aided actimeter (Actitrack, Panlab, Barcelona, Spain). This apparatus consisted of a 45 × 45 cm area, with infrared beams all around, spaced 2.5 cm, coupled to a computerized control unit that analyses the following parameters: (i) distance run in the actimeter (ambulation); (ii) mean velocity developed during this running; and (iii) time spent in fast (>5 cm s$^{-1}$) and slow (<5 cm s$^{-1}$) movements. Animals stayed in the actimeter for a period of 10 min, but measurements were only recorded during the final 5 min (first 5 min was used only for animal acclimatization).

*Hot-plate test.* Rats were placed on a hot-plate maintained at 52°C, and the latency to exhibit the first sign of pain (i.e. licking the hind paws or jumping) was measured for each animal. Animals not responding were removed after 30 s (cut-off time to avoid tissue damage).

## Determination of dopamine and glutamate concentrations

*Dissection procedure.* Coronal slices (around 500 μm thick) were obtained manually from brains at the level containing the caudate-putamen, globus pallidus and substantia nigra. Subsequently, these structures were dissected and homogenized in 20–40 vol of cold 150 mM potassium phosphate buffer, pH 6.8 and the dopamine and glutamate content of each homogenate was analysed. An aliquot of each homogenate was used to determine the protein concentration (Lowry *et al.*, 1951).

*Determination of dopamine content.* Dopamine content was determined using HPLC with electrochemical detection (Lastres-Becker *et al.*, 2005; González *et al.*, 2006). Briefly, homogenates were diluted in ice-cold 0.2 N perchloric acid containing 0.2 mM sodium disulphite and 0.45 mM EDTA, and dihydroxybenzylamine (20 ng·mg$^{-1}$) was added as an internal standard. The diluted homogenates were then centrifuged, and the supernatants were injected into the HPLC system. This system consisted of a Spectra-Physics 8810 pump and an RP-18 column (Tracer Excel 120 ODSB; 150 mm, 4.6 mm, 5 μm particle size; Teknokroma, Barcelona, Spain). The mobile phase, previously filtered and degassed, was a solution 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$^{-1}$. The effluent was monitored with a coulochemical detector (Coulochem III, 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 on a Spectra-Physics 4290 integrator from the analytical cell #2, with a sensitivity of 50 nA (10 pg per sample). Dopamine levels were calculated from areas under the peaks using the comparison with the internal standard area. Values are expressed as ng·mg$^{-1}$ of protein.

*Determination of glutamate content.* Homogenates were used for the analysis of glutamate content using a commercial Glutamate Assay Kit (#K629-100, BioVision, Mountain View, CA, USA) and following the instructions provided by the manufacturer. Values were calculated as nmol·mg$^{-1}$ of protein and expressed as % of controls.

### Immunohistochemical procedures

Coronal sections (20 µm thick) were obtained from the previously frozen brains with a cryostat and collected on gelatin-coated slides. Sections were fixed in fresh 4% paraformaldehyde prepared in 0.1 M PBS, pH 7.4. After being washed in PBS, sections were incubated overnight at room temperature with the primary antibodies: (i) monoclonal anti-tyrosine hydroxylase (Chemicon-Millipore, Temecula, CA, USA) used at 1:500; and (ii) monoclonal anti-rat CD11b clon OX-42 (Serotec, Bionova Científica, Madrid, Spain) used at 1:50. Next, sections were incubated with the secondary antibody, biotinylated horse anti-mouse antibody (Vector Elite, Burlingame, CA, USA), used at 1:200, followed by streptavidin incubation. We used Alexa Fluor-488 conjugated streptavidin (1:200, Molecular Probes, Eugene, OR, USA) for tyrosine hydroxylase immunostaining, and Alexa Fluor-546 conjugated streptavidin (1:200, Molecular Probes) for OX-42 immunostaining. Sections were then stained with 45 µM Hoechst 33342 and the slides were coverslipped with Vectashield mounting medium (Vector Elite, Burlingame, CA, USA). 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. In the case of tyrosine hydroxylase immunostaining in mouse sections, we used Mouse on Mouse Immunodetection Kit (Vector Elite), following the instructions provided by the manufacturer, to avoid secondary antibody cross-reaction. A Nikon Eclipse 90i microscope and a Nikon DXM 1200F camera were used for slide observation and photography. The magnitude of tyrosine hydroxylase or OX-42 immunostaining in the substantia nigra (both sides) for each animal was determined with NIH Image Processing and Analysis software (ImageJ; NIH, Bethesda, MD, USA) using three to six randomly selected sections, separated by 200 µm, and observed with a 5× objective. In all sections, the same area of substantia nigra pars compacta was analysed. Background signal was determined for each section in a small area near the one being examined. Analyses were always conducted by experimenters who were blinded to all animal characteristics.

Immunohistochemical analysis of $CB_2$ receptor (also in those immunostainings for tyrosine hydroxylase carried out in LPS-lesioned mice) was performed in paraformaldehyde-perfused brains instead of frozen brain sections. Fixed brains were sliced in a cryostat (30 µm thick) and collected on gelatine-coated slides. Sections were incubated overnight at room temperature with polyclonal anti-$CB_2$ receptor (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Next, sections were incubated with an Alexa-Fluor 594 chicken anti-goat secondary antibody conjugate (1:200; Molecular Probes). A Nikon Eclipse 90i microscope and a Nikon DXM 1200F camera were used for slide observation and photography.

### Data analysis

Data were subjected to one-way ANOVA followed by the Student–Newman–Keuls test, using Graph-Pad software (La Jolla, CA, USA) (version 4.0).

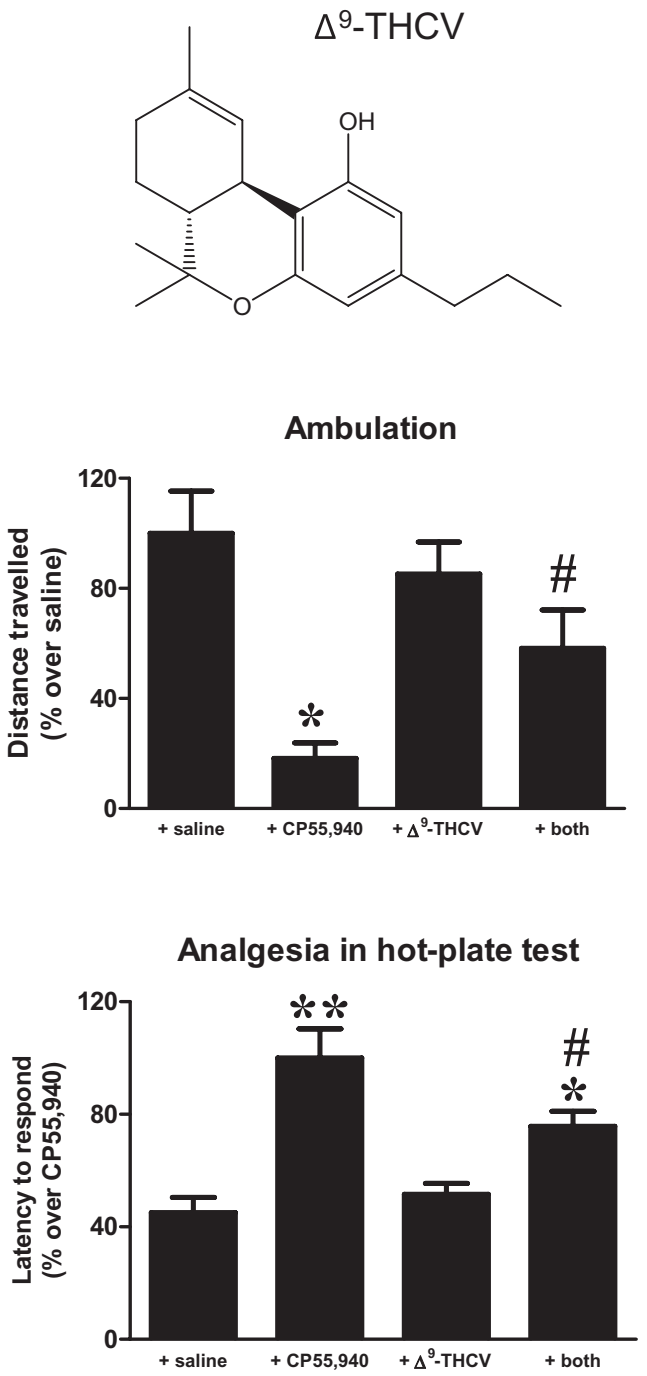
## Results

### Antagonism by Δ9-THCV of hypokinetic and analgesic effects of CP55,940

Figure 1 shows data obtained in experiments directed at establishing whether Δ9-THCV (2 mg·kg$^{-1}$) behaves as a $CB_1$ receptor antagonist *in vivo* in rats, as previously demonstrated in studies conducted in mice (Pertwee *et al.*, 2007; Bolognini *et al.*, 2010). As expected, the potent cannabinoid receptor agonist CP55,940 reduced ambulation in the actitrack [$F(3,21) = 7.983$, $P < 0.005$; Figure 1], altered other motor parameters in the same direction (data not shown) and increased the latency of the response by animals to a noxious stimulus in the hot-plate test [$F(3,22) = 13.84$, $P < 0.001$; Figure 1], two effects that are thought to be induced by the activation of $CB_1$ receptors. The administration of Δ9-THCV did not produce any effect by itself, but it partially attenuated the effects of CP55,940 in both assays (Figure 1), thus supporting the notion that Δ9-THCV also behaves as a $CB_1$ receptor antagonist in rats at the dose of 2 mg·kg$^{-1}$.

### Effect of Δ9-THCV on motor inhibition and dopamine and glutamate anomalies in 6-hydroxydopamine-lesioned rats

Figures 2 and 3 show the effects of an acute injection of Δ9-THCV (2 mg·kg$^{-1}$) or rimonabant (0.1 mg·kg$^{-1}$) on motor inhibition (lower ambulation, mean velocity and time spent in fast movements and increased time spent in slow movements) and dopamine and glutamate anomalies caused by lesioning with 6-hydroxydopamine (i.c.v). In this model, striatal dopamine content is significantly reduced (fivefold) 14 days post lesion, although this is accompanied by small motor anomalies, in accordance with human cases of parkinsonism where the appearance of motor symptoms occurs only after a loss of dopaminergic neurones greater than 50% (Nagatsu and Sawada, 2007). This was also observed in the present study (see Figures 2 and 3). Our behavioural data indicated that both Δ9-THCV and rimonabant were equally effective in enhancing ambulation [$F(3,18) = 4.14$, $P < 0.05$], mean velocity [$F(3,18) = 4.22$, $P < 0.05$] and, to a lesser extent, time spent doing fast movements [$F(3,19) = 1.992$, $P = 0.156$], reaching in all cases values similar to those exhibited by sham-operated animals (Figure 2). In addition, Δ9-THCV and




## Figure 1

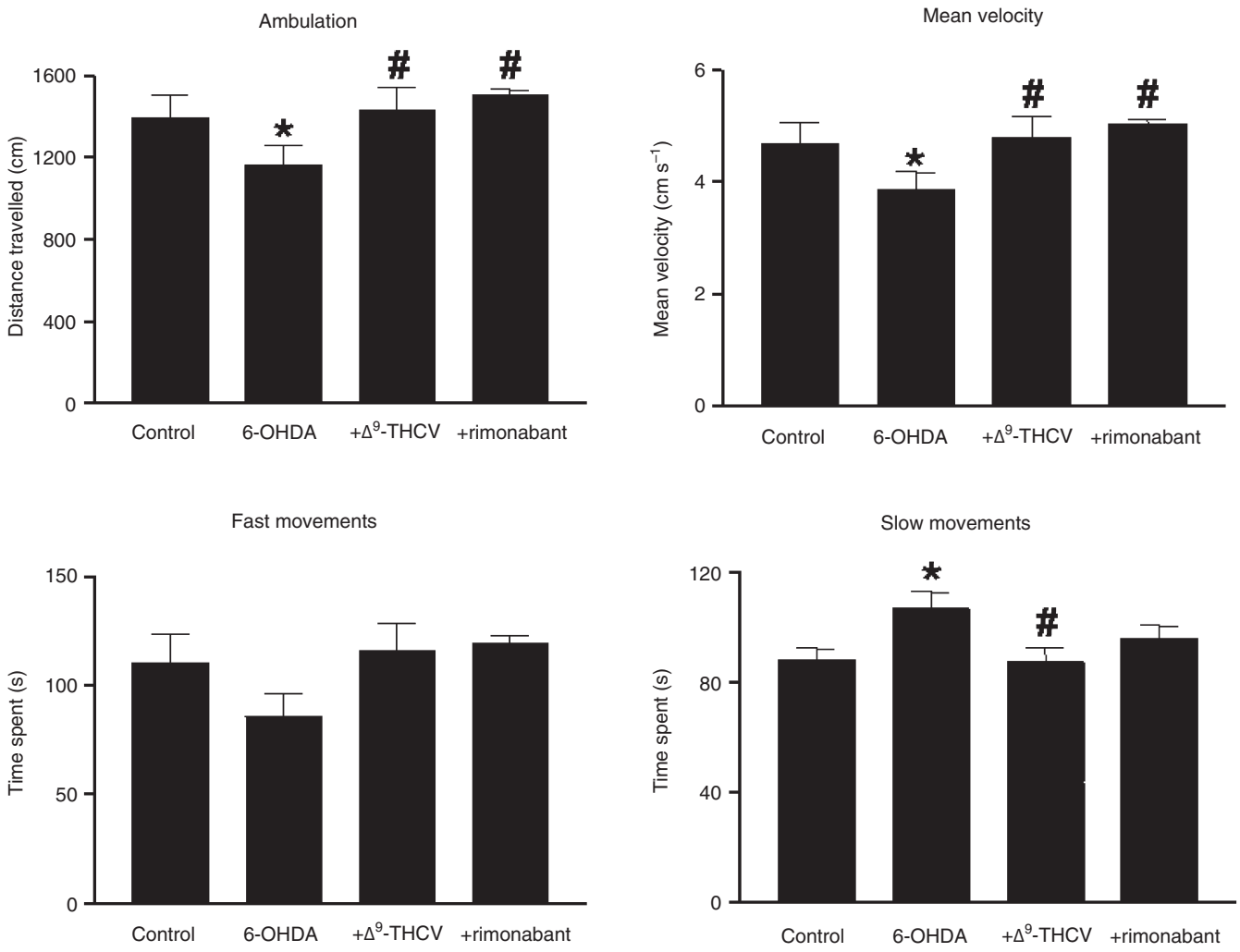
Effect of a single i.p. injection of $\Delta^9$-THCV (2 mg·kg$^{-1}$; its structure is shown in the top panel) on CP55,940-induced motor inhibition and analgesia (measured in the hot-plate test) in naïve rats. Values were normalized versus the group having the maximal response and are expressed as means ± SEM ($n$ = 5–6 rats per group). Data were subjected to one-way ANOVA followed by the Student–Newman–Keuls test (*$P$ < 0.05, **$P$ < 0.005 vs. control and $\Delta^9$-THCV-treated animals; #$P$ < 0.05 vs. CP55,940-treated animals).

rimonabant also reduced the large amount of time that these animals spent displaying slow movements [$F(3,21)$ = 3.037, $P$ < 0.05; Figure 2].

The effect of $\Delta^9$-THCV on behaviour was not associated with an increase in dopamine levels in the striatum, which, as mentioned above, is significantly reduced by lesioning with 6-hydroxydopamine [$F(2,16)$ = 8.30, $P$ < 0.005; Figure 3]. However, it was paralleled by signs of an increase in the glutamate content of the striatum, although this effect did not reach statistical significance [$F(2,17)$ = 2.786, $P$ = 0.094; Figure 3], as well as by a significant reduction of this chemical in the substantia nigra [$F(2,17)$ = 3.991, $P$ < 0.05; Figure 3]. No changes in glutamate levels were found in the globus pallidus (Figure 3). In all three structures, values of glutamate levels in control animals were as expected (striatum: 42.1 ± 5.2 nmol·mg$^{-1}$ protein; globus pallidus: 50.1 ± 5.9 nmol·mg$^{-1}$ protein; and substantia nigra: 36.8 ± 2.6 nmol·mg$^{-1}$ protein) from previous findings (González *et al.*, 2006). Values were normalized for graphical presentation (see Figure 3).

### *Effect of $\Delta^9$-THCV on tyrosine hydroxylase immunostaining in the substantia nigra of 6-hydroxydopamine-lesioned rats or LPS-lesioned mice*

Figure 4 shows the effects of chronic treatment with $\Delta^9$-THCV (2 mg·kg$^{-1}$; 14 days), or with a CBD-enriched botanical extract (equivalent to 3 mg·kg$^{-1}$ of pure CBD; 14 days), on the damage in nigral neurones caused by 6-hydroxydopamine (i.c.v.). These data indicate that the loss of nigrostriatal dopaminergic neurones, determined by tyrosine hydroxylase immunostaining in the substantia nigra, was reduced after chronic administration of $\Delta^9$-THCV [$F(3,18)$ = 4.869, $P$ < 0.05; Figure 4]. $\Delta^9$-THCV also attenuated the enhanced microglial activation caused by 6-hydroxydopamine, as measured by OX-42 immunostaining in the substantia nigra [$F(3,18)$ = 31.63, $P$ < 0.0001; Figure 4]. Both effects were also seen, to an even greater extent, with CBD-enriched botanical extract (Figure 4), in accordance with previous data obtained with pure CBD (Lastres-Becker *et al.*, 2005; García-Arencibia *et al.*, 2007). This finding possibly indicates that the neuroprotective effects of $\Delta^9$-THCV in 6-hydroxydopamine-lesioned rats are due more to its antioxidant properties than to its ability to activate $CB_2$ receptors. Previous studies have indicated that these receptors do not appear to play an important role in nigrostriatal lesions caused by 6-hydroxydopamine (García-Arencibia *et al.*, 2007). We obtained further evidence for this by showing that immunostaining for $CB_2$ receptors in the substantia nigra of the lesioned side was very weak (only a few cells were labelled with the anti-$CB_2$ antibody; see Figure 5) and so not much different from that seen in the contralateral non-lesioned side of each animal (Figure 5). In addition, lesioning $CB_2$ receptor–deficient mice with 6-hydroxydopamine led to a loss of tyrosine hydroxylase immunostaining in the substantia nigra that was of the same magnitude (approximately 50%) as that observed in wild-type animals (Figure 5). For these two last experiments, we used a unilateral lesion with 6-hydroxydopamine as this is much more appropriate for evaluating the degree of response against this neurotoxin, since this procedure allows the contralateral structures to serve as controls for the different




## Figure 2

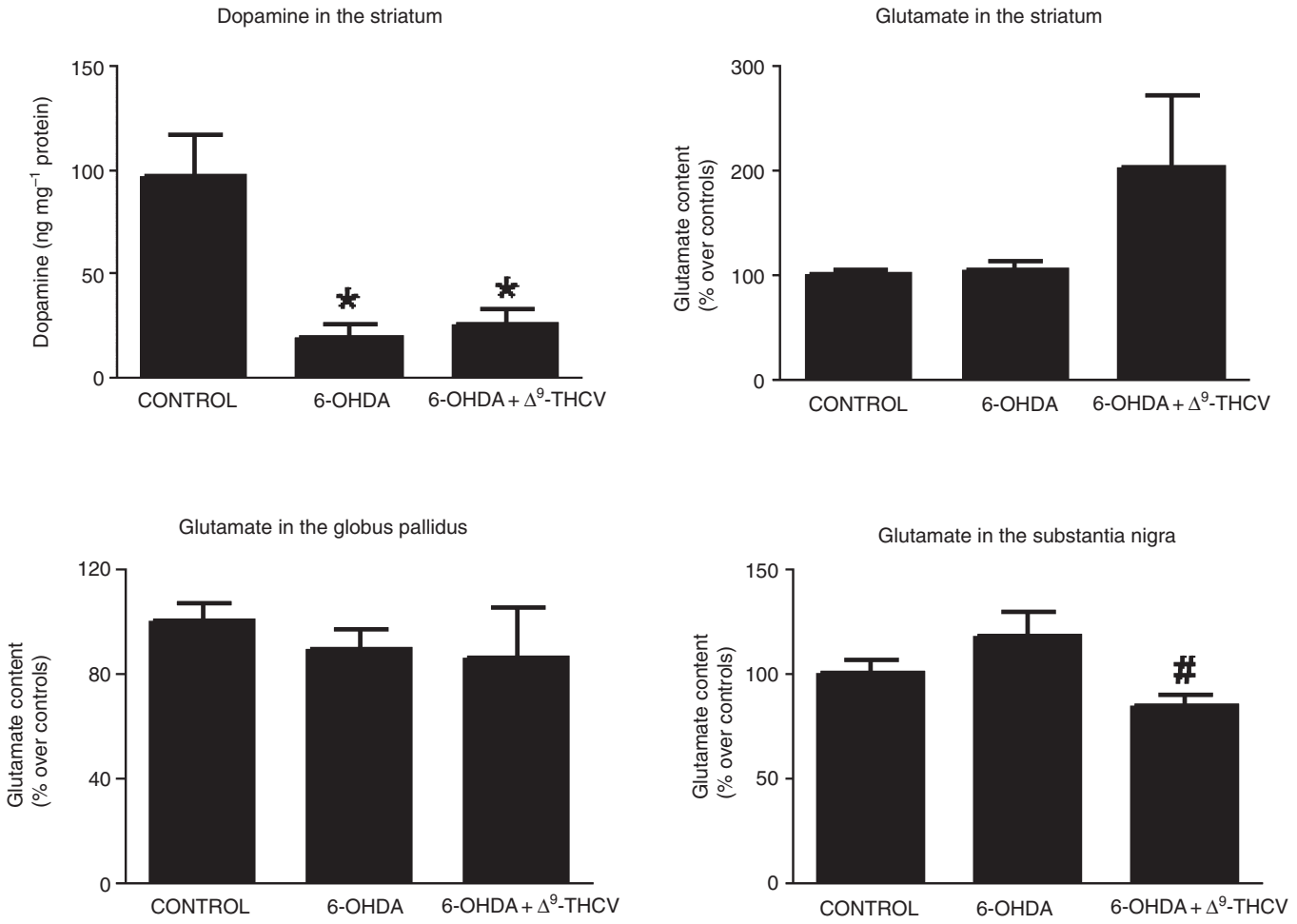
Motor parameters measured using a computer-aided actimeter. Rats were injected i.c.v. with 6-hydroxydopamine or saline (control rats) and subjected to a single i.p. injection of $\Delta^9$-THCV (2 mg·kg$^{-1}$), rimonabant (0.1 mg·kg$^{-1}$) or vehicle two weeks later. Values are means ± SEM ($n$ = 5–6 rats per group), and the data were subjected to one-way ANOVA followed by the Student–Newman–Keuls test (*$P$ < 0.05 vs. controls; #$P$ < 0.05 vs. 6-hydroxydopamine-lesioned animals).

analyses (up-regulation of $CB_2$ receptors and loss of tyrosine hydroxylase immunostaining).

Since 6-hydroxydopamine induces effects that do not appear to be counteracted by $CB_2$ receptor activation, we conducted additional experiments using a model of PD in which $CB_2$ receptors are expected to have a greater protective role. More specifically, mice were administered LPS by a unilateral intrastriatal injection, and it was found that immunostaining for $CB_2$ receptors in the lesioned substantia nigra was more intense than in the contralateral non-lesioned structure (Figure 6). These were presumably $CB_2$ receptors located in microglial cells labelled with IBA-1 (data not shown). These animals showed a significant reduction in tyrosine hydroxylase immunoreactivity [$F(3,17) = 4.73$, $P < 0.05$; see Figure 6] that reached approximately 32%, in line with data published by Hunter *et al.* (2009) who used different amounts of LPS. The administration of $\Delta^9$-THCV (2 mg·kg$^{-1}$, i.p.) to LPS-lesioned animals was associated with the preservation of tyrosine hydroxylase–positive neurones, as indicated by immunostaining for this enzyme (Figure 6). The same effect was also observed with the selective $CB_2$ receptor agonist HU-308 (Figure 6), thus supporting the involvement of $CB_2$ receptors in this PD model. This was further supported by the finding that $CB_2$ receptor–deficient mice were more vulnerable to LPS-induced lesions than wild-type animals (Figure 6).

## Discussion

Several cannabinoids have been found to display signs of therapeutic efficacy in animal models of PD, although their effects were limited to the alleviation of specific motor symptoms [e.g. rimonabant for akinesia (Fernández-Espejo *et al.*, 2005; González *et al.*, 2006; Kelsey *et al.*, 2009) or levodopa-induced dyskinesia (for review, see García-Arencibia

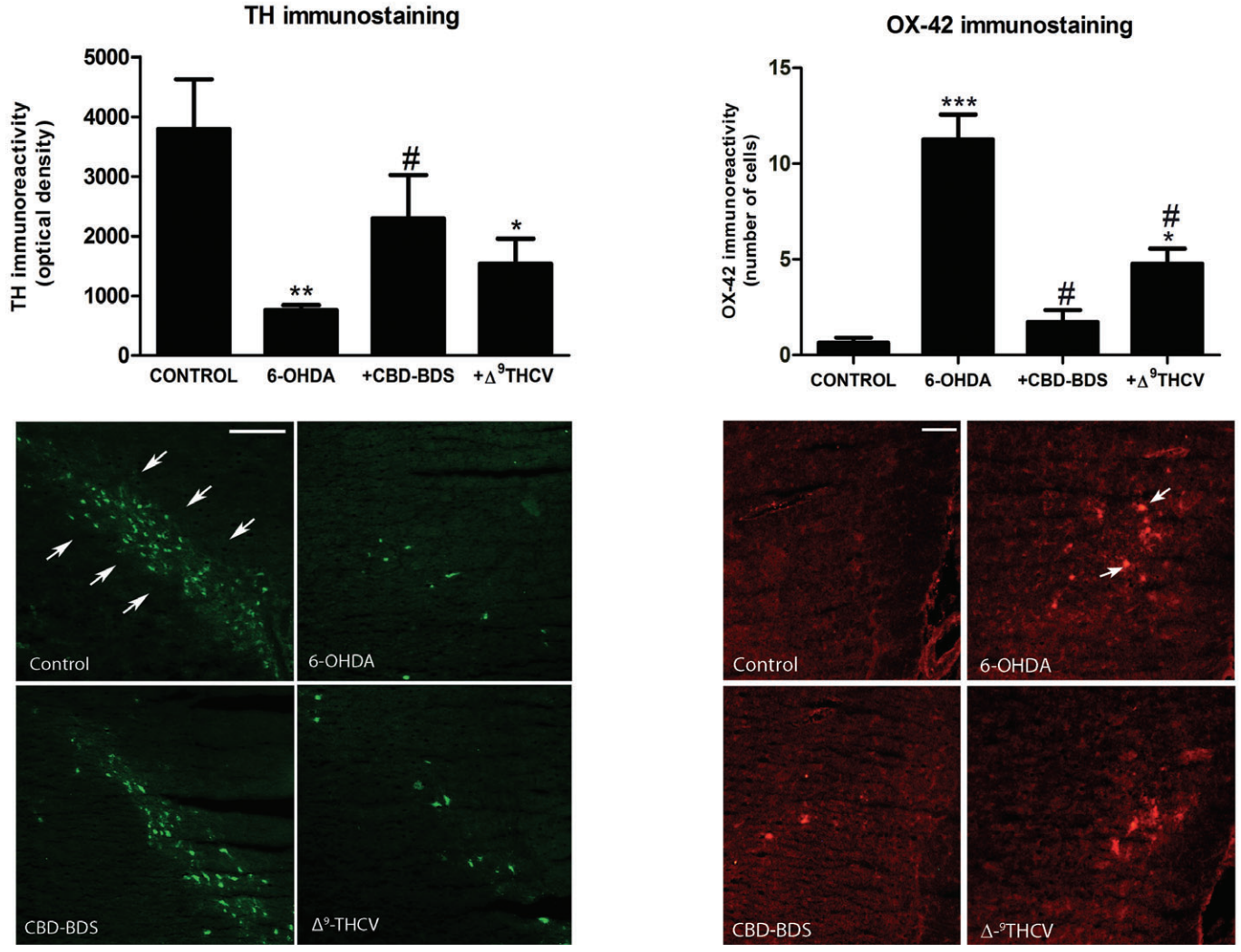



## Figure 3

Dopamine content in the striatum and glutamate concentrations in different basal ganglia structures of rats injected i.c.v. with 6-hydroxydopamine or saline (control rats) and subjected to a single i.p. injection of $\Delta^9$-THCV (2 mg·kg$^{-1}$) or vehicle 2 weeks later. Values are means ± SEM ($n$ = 5–6 rats per group), and the data were subjected to one-way ANOVA followed by the Student–Newman–Keuls test (*$P$ < 0.005 vs. controls; #$P$ < 0.05 vs. 6-hydroxydopamine-lesioned animals).

*et al.*, 2009), $CB_1$ receptor agonists for tremor (Sañudo-Peña *et al.*, 1999) and antioxidant cannabinoids (Lastres-Becker *et al.*, 2005; García-Arencibia *et al.*, 2007; Jiménez-Del-Rio *et al.*, 2008) or $CB_2$ receptor agonists (Price *et al.*, 2009) for the progression of nigral damage]. Classical anti-parkinsonian dopaminergic-replacement therapy also possesses the same disadvantage of inducing symptom relief without delaying disease progression (Pezzoli and Zini, 2010). Therefore, the search for novel compounds that will simultaneously ameliorate motor symptoms and delay progression of nigral damage represents an important challenge for this disease. In this study, we have examined a phytocannabinoid compound, $\Delta^9$-THCV, whose structural similarity to $\Delta^9$-THC and ability to block $CB_1$ receptors but activate $CB_2$ receptors (Thomas *et al.*, 2005; Pertwee *et al.*, 2007; Bolognini *et al.*, 2010), indicated *a priori* that it might meet this need both to relieve parkinsonian symptoms and to protect nigral neurones from death.

Here we have demonstrated that an acute injection of $\Delta^9$-THCV is efficacious in reducing motor inhibition in parkinsonian rats, with a potency equivalent to rimonabant. We also found that, as described previously for rimonabant (García-Arencibia *et al.*, 2008), these effects were dopamine-independent and associated with changes in glutamatergic transmission in key structures of the basal ganglia. For example, the administration of $\Delta^9$-THCV tended to elevate glutamate levels in the striatum, an effect that may be associated with a blockade of $CB_1$ receptors located in corticostriatal glutamatergic terminals, in accordance with the stimulant effects of rimonabant on glutamate release found previously by using *in vivo* microdialysis (García-Arencibia *et al.*, 2008) and with the well-known ability of $CB_1$ agonists to reduce the release of this transmitter (Adermark *et al.*, 2009). Any benefits resulting from this increase in glutamate might be mediated by glutamatergic receptor subtypes (e.g. group III metabotropic receptors) located in striatopallidal neurones that have been proposed to induce anti-parkinsonian effects (Ossowska *et al.*, 2007). It is also important to note that this stimulating effect on glutamatergic transmission is particularly marked in conditions of

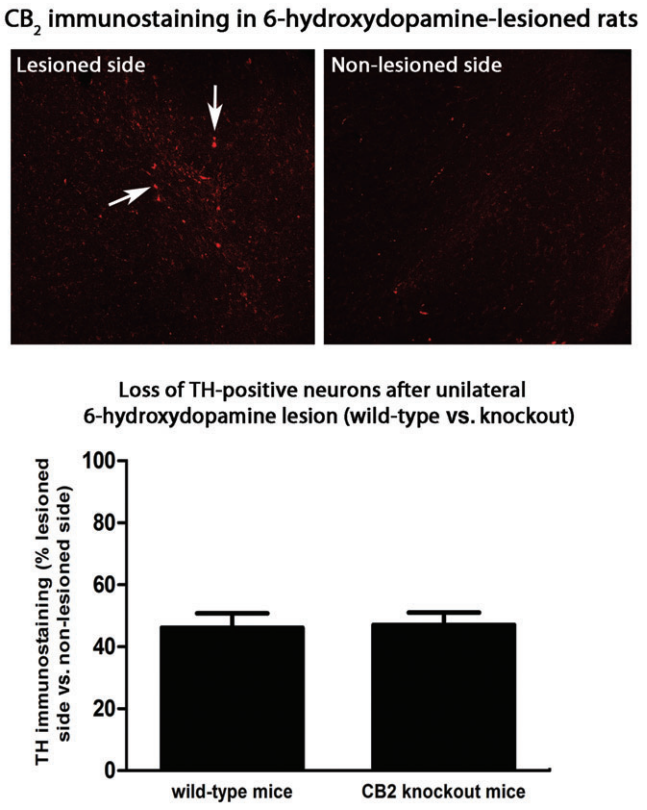



## Figure 4

Tyrosine hydroxylase and OX-42 immunoreactivity measured in the substantia nigra of rats injected i.c.v. with 6-hydroxydopamine or saline (control rats) and subjected to chronic i.p. treatment with Δ9-THCV (2 mg·kg$^{-1}$; 14 days), CBD-enriched botanical extract (4.63 mg·kg$^{-1}$, equivalent to 3 mg·kg$^{-1}$ of pure CBD; 14 days) or vehicle, starting 16 h after the i.c.v. injection. Values are means ± SEM ($n$ = 5–6 rats per group), and the data were subjected to one-way ANOVA followed by the Student–Newman–Keuls test (*$P$ < 0.05, **$P$ < 0.005, ***$P$ < 0.0005 vs. controls; #$P$ < 0.05 vs. 6-hydroxydopamine-lesioned animals). Representative tyrosine hydroxylase- and OX-42-immunostained sections of the substantia nigra are shown for the four experimental groups in the lower panels (scale bar = 100 μm for tyrosine hydroxylase and 50 μm for OX-42). Tyrosine hydroxylase– and OX-42-positive cells are indicated with arrows.

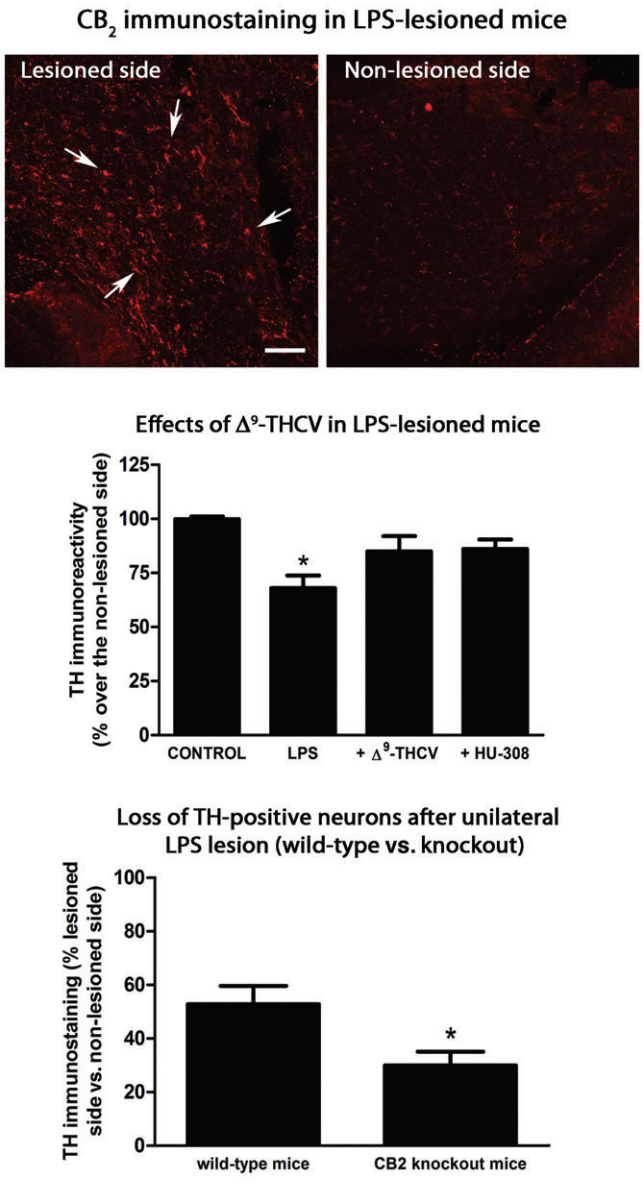
nigrostriatal dopaminergic denervation, being very modest in control animals, as described previously (García-Arencibia *et al.*, 2008). This might be because dopaminergic denervation of the striatum may particularly affect $CB_1$ receptors located in corticostriatal terminals, thus enabling a greater control of glutamate transmission by these receptors in conditions such as PD. Thus, such denervation was found some time ago, in experiments using autoradiography and *in situ* hybridization that do not allow cellular resolution, to be associated with an up-regulation of $CB_1$ receptors in different neuronal subpopulations within the striatum (Mailleux and Vanderhaeghen, 1993; Lastres-Becker *et al.*, 2001).

Glutamate levels in the substantia nigra of parkinsonian animals were reduced by Δ9-THCV. In our opinion, this was not the result of a direct action of Δ9-THCV on $CB_1$ receptors located in subthalamonigral terminals. Instead, it was most likely an indirect effect that resulted from blockade of $CB_1$ receptors located on striatopallidal terminals that, by enhancing GABA uptake (Maneuf *et al.*, 1996), would be expected to cause a reduction in the activity of these neurones and a subsequent decrease in their tonic inhibitory action on GABAergic neurones projecting from the globus pallidus to the subthalamic nucleus, resulting in a greater inhibition of glutamate release from subthalamonigral neurones. This would be expected to correct the excessive subthalamonigral activity underlying parkinsonian symptoms such as tremor (Nagatsu and Sawada, 2007), which, in our 6-hydroxydopamine-lesioned animals, was only evident as a




## Figure 5

Top panel: representative $CB_2$ receptor-immunostained sections of the substantia nigra of rats unilaterally injected with 6-hydroxydopamine. Scale bar = 100 μm. $CB_2$ receptor–positive cells are indicated with arrows. Bottom panel: tyrosine hydroxylase immunostaining measured in the substantia nigra of $CB_2$ receptor–deficient and wild-type mice unilaterally injected with 6-hydroxydopamine. Values are means ± SEM ($n$ = 5–6 mice per group) and are expressed as % of immunoreactivity in the lesioned side versus the non-lesioned side in the same animal. Data were subjected to Student's $t$-test.




## Figure 6

Top panel: representative $CB_2$ receptor-immunostained sections of the substantia nigra of mice unilaterally injected with LPS. Scale bar = 100 μm. $CB_2$ receptor–positive cells are indicated with arrows. Middle panel: tyrosine hydroxylase immunoreactivity measured in the substantia nigra of mice unilaterally injected with LPS or saline (control mice) and subjected to chronic i.p. treatment with $\Delta^9$-THCV (2 mg·kg$^{-1}$), HU-308 (5 mg·kg$^{-1}$) or vehicle (14 days), starting 16 h after the LPS injection. Values are means ± SEM ($n$ = 5–6 mice per group), and the data were subjected to one-way ANOVA followed by the Student–Newman–Keuls test (*$P$ < 0.05 vs. the remaining groups). Bottom panel: tyrosine hydroxylase immunostaining measured in the substantia nigra of $CB_2$ receptor–deficient and wild-type mice unilaterally injected with LPS. Values are means ± SEM ($n$ = 5–6 mice per group) and are expressed as % of immunoreactivity in the lesioned side versus the non-lesioned side in the same animal. Data were subjected to Student's $t$-test (*$P$ < 0.05 vs. wild-type animals).

trend towards an increase in glutamate content in the substantia nigra. Such a correction is fully concordant with the type of effect expected for an anti-parkinsonian agent.

The attenuation of motor inhibition via modulation of excitatory transmission is not the only beneficial effect that $\Delta^9$-THCV may provide in PD. Thus, given its classical phytocannabinoid structure, this compound may serve as an antioxidant agent with the same efficacy as CBD or $\Delta^9$-THC, two cannabinoids that have been demonstrated to be neuroprotective in 6-hydroxydopamine-lesioned rats (Lastres-Becker *et al.*, 2005; García-Arencibia *et al.*, 2007). In addition, $\Delta^9$-THCV is also a potent $CB_2$ receptor agonist that may share the ability of other agonists of this receptor to protect against nigrostriatal cell loss in MPTP-lesioned mice (Price *et al.*, 2009). However, in our previous studies conducted in 6-hydroxydopamine-lesioned rats, we found that selective $CB_2$ agonists did not have protective effects (García-Arencibia *et al.*, 2007), and we have found here that $CB_2$ receptors are poorly up-regulated in response to damage in these animals. This may be related to the fact that inflammation is a

secondary event in 6-hydroxydopamine-lesioned animals, as reflected by the modest levels of OX-42 immunoreactivity found in the substantia nigra in the model used in our present study. With this idea in mind, we investigated whether $\Delta^9$-THCV induces neuroprotective effects when administered daily for 14 days to 6-hydroxydopamine-lesioned rats. Our data strongly demonstrated that $\Delta^9$-THCV partially reduced 6-hydroxydopamine-induced nigral damage as shown by measuring tyrosine hydroxylase immunostaining and by the ability of this cannabinoid to attenuate OX-42 immunoreactivity (indicative of reactive microgliosis). Both responses were also elicited by CBD-enriched botanical extract, in accordance with previous data obtained with pure CBD (Lastres-Becker *et al.*, 2005; García-Arencibia *et al.*, 2007). The effects of CBD, which does not bind $CB_2$ receptors except at high concentrations, were in general of greater magnitude than the effects of $\Delta^9$-THCV, thus supporting the hypothesis that the antioxidant activity of both phytocannabinoids is possibly the key mechanism providing neuroprotection in this model of PD. This hypothesis is also supported by our observation that 6-hydroxydopamine lesions were of similar magnitude in $CB_2$-deficient and wild-type mice, and by the above-mentioned poor up-regulation of $CB_2$ receptors in the substantia nigra of 6-hydroxydopamine-lesioned rats. In this context, our data also suggest that the combined administration of both phytocannabinoids may enhance their neuroprotective properties in 6-hydroxydopamine-lesioned animals, while retaining the symptom-relieving effects of $\Delta^9$-THCV alone. On the other hand, although the neuroprotective effects of $\Delta^9$-THCV do not appear to be related to its ability to activate $CB_2$ receptors in the model of 6-hydroxydopamine used here, we assume that this property may be more important in pro-inflammatory models of PD, as well as an important factor to consider when developing a $\Delta^9$-THCV-based therapy for PD patients where inflammation is also a key element in the pathogenesis. To test this hypothesis, we used the LPS-lesioned mice model of PD where dopaminergic cell death is caused predominantly by inflammatory events. The substantia nigra of these animals exhibited a more intense up-regulation of $CB_2$ receptors compared both with the contralateral non-lesioned structures and to 6-hydroxydopamine-lesioned rats, and, accordingly, the treatment with $\Delta^9$-THCV also led to neuroprotection. We assume that this neuroprotective effect was a result of $\Delta^9$-THCV-induced activation of $CB_2$ receptors, as it was mimicked in the same model of PD by HU-308, a selective $CB_2$ receptor agonist, whereas mice lacking $CB_2$ receptors appeared to be particularly vulnerable to LPS lesion.

## Conclusion

In summary, given its antioxidant properties and its ability to activate $CB_2$ but block $CB_1$ receptors at a dose of 2 $mg \cdot kg^{-1}$, $\Delta^9$-THCV seems to have an interesting and therapeutically promising pharmacological profile. Thus, in contrast to other phytocannabinoids that have been investigated to date, it shows promise both for the treatment of disease progression in PD and for the relief of PD symptoms. This represents an important advance in the search for potential novel anti-parkinsonian agents, since $\Delta^9$-THCV administered alone or in combination with CBD may provide a much needed improved treatment for PD.

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## Conflict of interest

Authors have formal links with GW Pharmaceuticals that funds some of their research.

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