Antidyskinetic Effect of L-DOPA/Rimonabant or L-DOPA/Capsazepine Oral Co-administration in a Rat Model of Parkinson Disease.

Behavioral and Cytological Evidences


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Abstract

The long-term use of L-DOPA for Parkinson disease is limited by the development of L-DOPA-induced dyskinesias (LIDs). However, recent studies have suggested that pharmacological targeting of the endocannabinoid system may provide a potential valuable therapeutic tool to suppress these LIDs. Therefore, using the 6-OHDA lesion in rats, we evaluated the ability of rimonabant or capsazepine with the addition of L-DOPA in: (1) the severity of LIDs, the dyskinetic effects were assessed using measures of abnormal involuntary movements (AIMs); (2) the protection of dopaminergic cell loss; and (3) in the cytological differences between treatments by means of analyzing the number of dendritic spines of the striatal medium-size spiny neurons and the neuropile preservation. Oral co-administration of each antagonist with L-Dopa significantly decreased LIDs. Our data demonstrate that co-administration of L-DOPA with CB1 or TRPV1 receptors antagonists result in a very efficient treatment to reduce AIMs through the conservation of some functional dopaminergic cells, which in turn imply the well-preserved synaptology of a less denervated striatum. Thus, consistent with other reports, cannabinoid antagonist-based therapy would not only be aimed at alleviating specific motor symptoms, but also at delaying/arresting the degeneration of striatal and substantia nigra compacta cells.

Keywords: L-DOPA, Dyskinesia, Parkinson disease experimental model, endocannabinoid system antagonists, CB1, TRPV1, ultrastructure, cytology

Introduction

Parkinson disease (PD) is a chronic and progressive neurodegenerative disorder of essentially unknown etiology firstly described by James Parkinson more than 180 years ago and now affecting tens of millions of people worldwide, with an associated high socioeconomic burden. The clinical features of the disease are represented by poverty of voluntary movements (akinesia), slowness and impaired scaling of voluntary movement (bradykinesia), muscle rigidity and limbs tremor at rest. These symptoms seem to represent the downstream effect of a pathological cascade resulting in the degeneration of midbrain dopaminergic (DAergic) neurons of the substantia nigra pars compacta (SNc) projecting to the striatum, the main input station of the basal ganglia neural circuit [62,63]. The discovery of dopamine (DA) deficiency in PD and the subsequent introduction of a replacement therapy with the DA precursor L-3,4-dihydroxyphenylalanine (L-DOPA) initially revolutionized the management of the disease [13]. Unfortunately, motor fluctuations and dyskinesias complicate L-DOPA treatment in most patients (>90%) within 5–10 years of treatment.
initiation. L-DOPA-induced dyskinesias (LIDs) are characterized by choreiform and dystonic movements and are classified according to their temporal profile as “peak-dose” (occurring at peak L-DOPA concentration in the brain), “diphasic” (at the beginning and end of dosing) and “off” dyskinesias (when L-DOPA concentration is low) [33, 85].

In rats, LID can be modeled via intracerebral injection of the neurotoxin 6-hydroxydopamine (6-OHDA), which damages the nigrostriatal pathway followed by chronic administration of low doses of L-DOPA, which causes characteristic abnormal involuntary movements (AIMs) and dyskinesias [69]. Hence, since L-DOPA treatment induces motor disturbances after 5-10 years, other treatments targeting non-DAergic systems have been proposed for the management of PD [64]. Thus, while PD is predominantly associated with the loss of DA, it is becoming widely accepted that this disease is also associated with disruptions in non-DAergic mechanisms [10]. A signaling system, which is becoming increasingly implicated in PD is the endocannabinoid system (ECB) [45, 66].

The major components of the ECB system are two endogenous lipids, -N-arachidonoylthanolamine or anandamide (AEA) and 2-arachidonyl-glycerol (2-AG), which are specific ligands of G-protein-coupled receptors named CB1 and CB2 receptors, respectively [24, 75, 76, 82]. Specifically, the CB1 receptor and its main endogenous ligands, AEA [23, 24] and 2-AG [101], are known to be present in high concentrations in the basal ganglia [95]. CB1 receptors are expressed on the presynaptic terminals of the striatonigral and striatopallidal neurons, as well as on the presynaptic terminals of corticostriatal neurons, and are supposed to exert a tonic inhibitory effect on these neurons via retrograde signaling from postsynaptic neurons [80, 92, 112]. CB1 receptors are also expressed on striatal interneuron subtypes containing parvalbumin, nitric oxide synthase, and Choline acetyltransferase [43]. Therefore, normal motor function is mediated by an extremely balanced signaling loop consisting of GABAergic striatonigral and striatopallidal projections, glutamatergic corticostriatal projections, and striatal interneurons under the tonic regulatory influence of nigrostriatal DA as well as ECBs [110] in order to modulate basal ganglia neural network dynamics and long-term forms of synaptic plasticity [25]. There is a growing body of evidence to suggest that ECB system is altered in both parkinsonian patients [58, 87, 93] and in experimental models of PD [14, 28, 35, 70, 111].

On the other hand, the transient receptor potential (TRP) receptors are ligand-gated ion channels that generate a cation inward flow upon activation [88]. Experimental evidence indicates that cannabinoids and ECBs directly interact with at least five distinct TRP channels, among which the vanilloid receptor VR1 (TRPV1) [16] emerges as the best-characterized “ionotropic” cannabinoid receptor [1]. This receptor is activated by vanilloids, such as capsaicin [15, 61], endogenous ligands, including AEA [26, 61] and N-arachidonoyl-dopamine [57]. TRPV1 receptors are present in the striatum, globus pallidus and substantia nigra [20, 78, 79]. In the latter region, they affect the excitability of DAergic neurons by
modulating excitatory inputs from glutamatergic terminals [73, 83], suggesting that TRPV1 might be involved in the control of movement [37, 73]. In support, treatment with TRPV1 agonist capsaicin decreases DA release in the striatum [53, 68] and decreases motor activity [26, 65]. Furthermore, pharmacological modulation of these receptors has been reported to influence striatum-dependent functions [21], and to modulate motor symptoms originating from striatal dysfunction [65, 105]. In the striatum, TRPV1 receptors co-localize with CB1 receptors [20, 27], suggesting a close functional interaction between these receptors.

It has been mentioned that early presymptomatic phases in PD are associated with down-regulation/desensitization of CB1 receptors [39, 41, 45], since the activation of CB1 receptors inhibits glutamate release, thus, it is possible that down-regulation/desensitization of these receptors observed in PD is associated with enhanced glutamate levels, and excitotoxicity plays an essential role in contributing to disease progression [45]. At later stages, a significant up-regulation of CB1 receptors was found in PD, which is caused by adaptive responses and is also compatible with the akinetic profile of these patients [37, 45]. It has also been reported that GABAergic medium size spiny neurons contain large number of CB1 cannabinoid receptors [55]. Activation of these receptors causes presynaptic inhibition of GABA release in vitro [7, 25, 37, 48, 102] and profoundly affects motor behaviors in vivo [14, 50, 74, 99]. Moreover, the interdependency between CB1 and DA receptors and their high abundance in the striatal system has led to the hypothesis that targeting the CB1 receptor could be of value to improve motor deficits in neurodegenerative motor diseases such as PD [71]. Thus, abnormalities in DA signaling, as reported in PD and related animal models, may disrupt this feedback mechanism [40] and lead to a functional state characterized by motor disturbances [47].

Experimental evidences suggest that the ECB transmission is increased in the basal ganglia of humans affected by PD [93] and in experimental models of this disease [14, 28, 52], thus the blockade of cannabinoid CB1 or TRPV1 receptors might be beneficial to alleviate PD symptoms. In fact, evidence from nonhuman primates and rodents has shown that the administration of the antagonist SR141716 (Rimonabant) improves motor symptoms in models of PD [12, 36, 49, 103, 108], and direct activation of CB1 receptors significantly attenuated L-DOPA-induced AIMs and the elevation of brain AEA via pharmacological blockade of its catabolism produced an anti-dyskinetic effect in the presence of TRPV1 antagonist capsazepine [81]. Altogether, the above data support the concept of targeting the CB1 and TRPV1 systems in the treatment of movement disorders, such as LID.

Dyskinesia is a highly disabling clinical manifestation of PD induced by the long term use of L-DOPA therapy, it has been proposed the antagonism of CB1 or TRPV1 as an alternative for the regulation of the up-regulated cannabinoid system. Therefore, using the 6-OHDA-medial forebrain bundle (mfb) lesion, we
evaluated the ability of rimonabant (RIM) or capsazepine (CZP) with the addition of L-DOPA (LD) in: (1) the presence of LD-induced dyskinesia, the severity of dyskinetic effects were assessed using measures of abnormal involuntary movements (AIMs); (2) the protection of SNc DAergic cell loss; and (3) in the structural and ultrastructural differences between treatments by means of analyzing the number of dendritic spines of the striatal medium-size spiny neurons and the neuropile preservation.

**Material and methods**

The experiments were carried out in 30 male Wistar rats weighing 180–200 g at the beginning of the study. The rats were individually housed in hanging plastic cages under controlled light conditions (12 h light/h dark regime) and fed with Purina Rat Chow and water *ad libitum*. Body weight was recorded daily. The experimental protocol was conducted in accordance with the Animal Act of 1986 for Scientific Procedures. All efforts were made to minimize the number of animals used and their suffering.

*Stereotactic surgery and treatments.*

The rats were anesthetized with sodium pentobarbitone (35 mg/Kg i.p.) and placed in a stereotaxic apparatus. The rats were injected with 4 μl of a saline solution containing 8 μg of 6-OHDA (Sigma Chemical, USA) and 0.2 mg of ascorbic acid into the right mfb (n = 24) and sham lesion was made with vehicle (n = 6 (control group)). The injections were given over a 4-min period with a Hamilton syringe attached to a glass micropipette with a tip diameter of 20–50 μm. The stereotaxic coordinates were as follows: AP = −4 mm anterior of the ear bar; L = 1.4 mm lateral of bregma; V = −7.7 mm vertical of dura (according to [90]). After recovery from the anesthesia, the animals were returned to their home cages. Apomorphine (Sigma Chemical, USA; 0.25 mg/Kg i.p.) induced rotational behavior was tested one day after lesioning. Only those animals exhibiting more than 200 complete turns in a 30 min period were used [106]. One day after the test, 6 experimental animals were treated with 15-mg/kg LD (Sinemet® (Carbidopa-L-DOPA 25/250) 1:10 ratio), 6 were treated with 1-mg/kg rimonabant co-administrated with 15-mg/kg LD (LD/RIM), 6 with 1mg/kg capsazepine co-administrated with 15-mg/kg LD (LD/CZP) (Cayman Chemicals, USA). Sinemet tablets were crushed and the powder was dissolved in 10 ml tap water, then the corresponding antagonists dose were added to Sinemet solution and given orally with an insulin syringe for two months. The other 6 lesioned rats without treatment, as well as control animals, were kept for the same time. It must be taken into account that there is evidence that administration of DAergic agents to 6-OHDA-lesioned rats can affect the course of DAergic lesion, and agonists such as apomorphine can induce a priming effect enhancing the emergence of DAergic
hypersensitivity [56], so we decided not to use the apomorphine-induced circling behavior to evaluate motor recovery.

**AIMs Rating.**
AIMs were scored every 14 days (4 evaluations). LD-induced AIMs were scored according to a rat dyskinesia scale [17]. Rats were placed individually in transparent plastic cages and observed every 20th min, from 20 min before taking the dose to 180 min after taking the treatment (10 monitoring periods of 1 min each). Four subtypes of AIMs were classified according to their topographic distribution as locomotive, axial, forelimb, or orolingual. Enhanced manifestations of otherwise normal behaviors, such as rearing, sniffing, grooming and gnawing, were not included in the rating. AIM severity was assessed using the published method of Cenci and Lundblad [17], which assigns a score from 0 to 4 to each of the four AIM subtypes listed above according to the proportion of time/monitoring period during which the AIM is present. Borderline scores, such as 0.5, 1.5, 2.5, and 3.5, were allowed in order to increase the sensitivity of the evaluation.

**Video Recording.**
Performance during AIM analysis was video recorded using a Panasonic camcorder (SDR-H80 model). Representative still frames were captured from digital video recordings with the video editing software Final Cut Pro. Pictures were cropped and adjusted for color and brightness contrast in Adobe Photoshop but were not altered in any other way.

**Tissue Preparation.**
All rats were perfused via aorta under sodium pentobarbital anesthesia immediately after the two-months treatments via aorta, with saline solution followed by fixative containing 0.2% glutaraldehyde and 4% paraformaldehyde in 0.1M-phosphate buffer (PB). The brains were removed and placed in fixative solution for 1 hour.

**TH Immunocytochemistry.**
Coronal sections (50 μm) were obtained on a vibrating microtome through the mesencephalon for immunocytochemistry. Tyrosine hydroxylase (Chemicon International, Inc. CA, USA, 1: 1000) immunostaining with the ABC detection method (Vector Lab MI, USA) was performed for light microscope analysis. The analysis was conducted with a computer-assisted system (Image-Pro Plus, Media Cybernetics, L.P. Del Mar, CA, USA) connected by a CCD camera to Optiphot 2 microscope (Nikon, Japan). The number of TH-positive neurons was counted in 1500 μm² from 7 SNc sections of each animal [5].

**Golgi Method.**
Blocks from the striatum were cut into 90-μm- thick sections and processed for the rapid Golgi method [107]. The histological analysis consisted in counting the
number of dendritic spines in a 10-µm-long section from 5 secondary dendrites from 10 medium-size spiny neurons [5].

**Electron microscopy.**
Fragments from contralateral and ipsilateral striata were carefully taken. After washing in PB, the fragments were treated for 60 minutes with 1% osmium tetroxide in PB, washed for 30 minutes in PB, dehydrated with graded ethanol and flat-embedded in araldite. Ultrathin sections were collected, counterstained with uranyl acetate and lead citrate, and examined in a JEOL 100CX-II electron microscope.

**Ultrastructural analysis.**
Synapses were defined by the presence of a clear post-synaptic density facing at least three pre-synaptic vesicles. Ultrastructural analysis was performed in 50 randomly selected synaptic endings per striatum. In each synaptic bouton we observed all its membrane and organelle features, and we measured the diameter of the presynaptic bouton using two axes, which were perpendicular to each other and intersected at the center of the synaptic terminal (Figure 1); the diameter was measured directly from the electron microscope screen with a grid placed inside the eyepiece [4].

![Figure 1](image)

**Figure 1.** Synaptic ending (B) showing the two axes measured, establishing a synaptic contact with a dendritic spine (S).

**Statistical Analysis.** Dyskinesias were expressed as median of total AIMs score and analyzed using the Kruskal–Wallis test followed by the Dunnett’s multiple comparison test. The threshold for statistical significance was set at p < 0.05. One-way ANOVA was used to analyze the number of TH-immunoreactive cells, number of dendritic spines and synaptic ending diameter and expressed as mean ± SEM. Group differences were considered statistically significant at P < 0.05. When appropriate, post-hoc comparisons were made with the Tukey test. Analyses were performed by GraphPad Prism version 5.0 for Mac (Graph Pad Software, San Diego, CA, USA).
Results

After 2 months, neither clinical alterations nor significant weight changes were detected in the experimental animals compared with controls.

Abnormal Involuntary Movements (AIMs). Time Course and Overall Incidence AIMs. In order to get an overview of the development of dyskinesia in the different groups we carried out the summation of all subtypes of AIMs (axial + locomotive + limb + orolingual). Figure 2A depicts the total sum of the severity of all AIM's induced by LD in each experimental condition at different times of treatment. LD/RIM-treated rats presented less severe AIMs compared with the group treated only with LD. A similar effect showed LD/CZP-treated group, nevertheless the attenuation of AIMs induced by CZP persisted to 56 days. As shown in Fig. 2B (treatment day 42), the temporal manifestation of AIMs after a single dose of LD alone or LD co-administrated with antagonists was similar in all groups, resembling the time course of peak-dose dyskinesia in PD [33, 85]. Thus, AIM severity gradually increased during the first 20 min post-treatment, remained elevated for an additional 60 min, and then gradually returned to baseline between 80 and 140 min post-treatment. The AIMs severity is much more evident in the LD-only treated group, since the co-administration with RIM or CZP significantly reduce the AIMs severity.

Representation of AIM Subtypes. According to Cenci and Lundblad [17], the animals were evaluated on four different topographic subtypes of AIMs. Different AIM subtypes were mainly characterized in LD-only treated group and with less severity in the antagonist-treated groups (Fig. 3). The development of orolingual (3A) axial (3B), and forelimb (3C) AIMs during chronic drug treatment differed greatly among groups. Indeed, chronic treatment with LD alone produced increasingly severe AIMs affecting trunk, limb and orolingual muscles. However, from the first to the last testing session, the co-administration with LD/RIM and LD/CZP attenuate development of AIMs, being more effective LD/CZP co-administration. Moreover, LD/RIM- and LD/CZP- treated groups demonstrate less impaired motor behavior. It is important to stand out that LD/CZP-treated rats showed ipsilateral axial AIMs, sometimes more frequently than contralateral ones (data not shown). Finally, locomotive AIM was barely observed in all experimental groups, both LD-alone and antagonist-treated groups manifest turning behavior involving two members (axial AIM) and only locomotive movements concerning all four limbs are rated under this AIM category.
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Figure 2. Time Course and Overall Incidence AIMs. The three treated groups, 6-OHDA+LD, 6-OHDA+LD/RIM and 6-OHDA+LD/CZP confer certain susceptibility to dyskinesia during the course of the experiment, but the overall AIM severity is most pronounced in rats with 6-OHDA+LD treatment. (A) Time course of AIM development during the chronic LD and LD/antagonist treatments. Values give total (locomotive + axial + orolingual + limb AIMs) integrated AIM scores per testing session as group median scores. (*=P < 0.001 LD and antagonist-treated groups versus 6-OHDA-lesion; @ =P < 0.005 LD/RIM versus LD group; #=P < 0.005 LD/CZP versus LD treated group, Kruskal–Wallis followed by Dunn's multiple comparison test). (B) Time course of total AIM scores/monitoring period after a single treatment of LD or antagonists (treatment day 42).

Figure 3. Representation of AIM Subtypes. Integrated AIM scores were generated separately for orolingual (A), axial (B), and forelimb (C) AIMs. (*=P < 0.005 treatments versus 6-OHDA-lesion; @ =P < 0.005 LD/RIM versus LD group; #=P < 0.005 LD/CZP versus LD treated group, Kruskal–Wallis followed by Dunn's multiple comparison test).
**TH Immunocytochemistry**

The number of TH-positive neurons in the control group, both contra and ipsilateral SNc remained unaffected (94 ± 1.9 and 93 ± 1.7, respectively) (Figs. 4 and 5). In contrast, we found an important loss of TH-positive neurons in the SNc of 6-OHDA lesioned animals in both contralateral (73 ± 1.9) and ipsilateral (5 ± 1.6) SNc compare to controls as shown in figures 4 and 5, likewise, LD-treated rats (59 ± 1.0 and 6 ± 2.0 contralateral and ipsilateral, respectively), in contrast LD/RIM (78 ± 2.2 contralateral and 20.3 ± 1.2 ipsilateral SNc) and LD/CZP- (81 ± 1.8 contralateral and 18 ± 0.9 ipsilateral SNc) treated rats show significant bilateral loss of TH-positive cells comparing to control group, however, when compare to LD-only treated group we found significant differences in both sides. As shown in Fig. 5 both antagonists revealed a protective effect on DAergic cell survival, being more effective LD/RIM co-administration.

![Figure 4. TH-immunoreactive cell counts from the SNc. The data are presented as the mean ± SEM. A statistically significant decrease in TH-immunoreactive cells was detected in both, contralateral (C) and ipsilateral (I) SNc in the four experimental groups, however, LD/RIM and LD/CZP treatments showed an evident protection (* = P < 0.05 versus control group; @ = P < 0.05 versus LD-treated group; ANOVA test).](image-url)
Figure 5. Representative TH-immunostained from coronal sections containing the SNc of control, 6-OHDA, 6-OHDA+LD, 6-OHDA+LD/RIM and 6-OHDA+LD/CZP treated rats. Note the profound cell loss in the ipsilateral SNc in the four experimental groups, being more evident in 6-OHDA and LD-only treated ones, in contrast it is striking the cellular protection provided by the antagonists-treated groups (Magnification 10x).
**Histological analysis.**

Figures 6 and 7 show the effects of all the treatments on dendritic spine density. The spine densities in the control group, both contra and ipsilateral striatum remained unchanged (15.23 ± 0.36 and 14.7 ± 0.30, respectively (Figs. 6 and 7A)). In contrast, 6-OHDA lesion and LD treatment induced an important loss of dendritic spines in both striata (Figs. 6 and 7B and C), likewise, LD/CZP (Figs. 6 and 7D) and LD/RIM groups (Figs. 6 and 7E) show significant differences in the ipsilateral striatum compared to control group but in less proportion. When comparing the 3 treated groups, we found that contralateral striatum of both antagonist-treated groups show similar values of those found in control group striatum, Post hoc analysis showed a significant reduction on spine density in the LD-treated group compare to LD/CZP and LD/RIM treated groups.

**Figure 6.** Golgi stain analysis. Dendritic spines density of medium-size spiny neurons of the contralateral (C) and ipsilateral (I) striata after two months. (* = P < 0.05 versus control group; @ = P < 0.05 versus LD-treated group; ANOVA test).
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**Figure 7.** Dendritic spine density. Photomicrographs of representative Golgi-stained medium-size spiny neurons of the ipsilateral striatum with representative box of dendritic spine densities from, control group (A), 6-OHDA-lesion (B), 6-OHDA + LD-treatment (C), 6-OHDA + LD/RIM-treatment (D) and 6-OHDA + LD/CZP-treatment (E). Both, 6-OHDA lesion and LD-treatment induced an important decrease in total number of spines mainly in the ipsilateral striatum. In contrast, antagonist-treated groups show well-preserved dendritic spines density (Magnification 40X and 100X).

**Electron Microscopy.**
Control rats did not show any differences between both striata synaptic endings diameter and neuropile alterations after sham surgery (Figs. 8 A, B and 9A). As shown in Figure 8, the synaptic endings of the control group, the major axis presented an average of $696.8 \pm 9.4$ nm on the contralateral striatum and $700 \pm 9.6$ nm on the ipsilateral one; the minor axis mean was $474.9 \pm 9.6$ nm on the
contralateral and 477.0 ± 9.6 nm on the ipsilateral striatum. The 6-OHDA-lesioned group showed an evident increase in the size of synaptic boutons (1379.7 ± 18 and 980.3 ± 16.13 nm major and minor axis, respectively on the ipsilateral striatum), the same pattern was observed in the 6-OHDA + LD-treated group (1340.0 ± 13.20 and 966.0 ± 12.10 nm major axis and minor axis of the ipsilateral side, respectively) there were statistically significant differences in both groups comparing to control group (Figs. 8 A, B and 9 B, C). 6-OHDA + LD/RIM group showed fewer presynaptic buttons with edema (987 ± 12.00 nm and 765 ± 13 nm major and minor axis of the ipsilateral side, respectively), likewise LD-/CZP rats displayed fewer swollen synaptic endings (1078 ± 12 nm and 878 ± 8.98 nm major and minor axis of the ipsilateral striatum, respectively), showing significant differences comparing to 6-OHDA untreated and LD-only treated groups. The contralateral (non-lesioned side) of all experimental groups showed no significant differences compare to control group (Fig. 8 A, B). The neuropile of 6-OHDA and LD-only treated groups was severely altered (Figs. 9 B, C), in contrast, neuropile of LD/RIM and LD/CZP was well preserved with no evident alterations (Figs. 9 D, E).

Figure 8. Ultrastructural analysis. Synaptic ending diameter in ipsilateral (I) and contralateral (C) striata after stereotactic surgery and treatments, major (A) and minor (B) axes. (∗ = P < 0.05 versus control group; @ = P < 0.05 versus LD-treated group; ANOVA test).
Figure 9. Striatal ultrastructure. Electron micrographs from ipsilateral striatum neuropile. (A) In control group, the mean size of the synaptic buttons (b) was 700 X 696 nm, it can be observe that the neuropile is well preserved, (s) dendritic spine, (d) dendrite. (B) This image shows a swollen synaptic button (b) of 6-OHDA rat establishing a synaptic contact with a dendritic spine (s), note the altered mitochondria and some vacuoles within neuropile. (C) This image demonstrates swollen pre-synaptic ending (b) of LD-treated rat establishing a synaptic contact with a dendritic spine (s), this rats also showed severe neuropile alterations. (D) LD/RIM and (E) LD/CZP, both, synaptic buttons and neuropile of antagonist-treated groups was well preserved similar to control group, (b) synaptic bouton, (s) dendritic spine, (d) dendrite. Bar 0.2μm
Discussion

The data of this study demonstrate that co-administration of L-DOPA with CB1 or TVPR1 receptors antagonists result in a very efficient treatment to reduce AIMS through the conservation of some functional SNc DAergic cells, which in turn imply the well-preserved synaptology of a less denervated striatum. Thus, cannabinoid antagonist-based therapy would not only be aimed at alleviating specific motor symptoms, but also at delaying/arresting the degeneration of striatal and SNC cells.

AIMs.
The evidence of an increase in the ECB transmission in the basal ganglia in PD patients and animal models of this disease [52, 93] supports the potential of Rimonabant or other CB1 receptor antagonists to alleviate PD symptoms. The data found in this work confirm this hypothesis, since we showed that blockade of CB1 receptors significantly attenuated 6-OHDA-induced hypokinesia. Moreover, the finding that the combination of 1 mg/kg Rimonabant and 15 mg/kg L-DOPA reduce the severity of LID appears to be an important demonstration that a cannabinoid antagonist can be adjunctively therapeutic in an animal model of PD. This finding is consistent with the intimate linkage between the DA and ECB systems within the basal ganglia [48, 74, 87] and suggests that adjunctive use of a cannabinoid antagonist might enable a reduction of the dose and, therefore, the side effects of L-DOPA needed to treat PD.

On the other hand, pharmacological blockade of TRPV1 unmasked the antidyskinetic effect of the fatty acid amide hydrolase (FAAH) inhibitor URB597 in a rat model of PD [81]. The existence of a crosstalk between CB1 and TRPV1 is evidenced by studies showing that CB1 stimulation can alter the functional state of TRPV1 [37, 51, 60, 61, 68, 81, 83, 89, 100], thus we conclude that after blocking CB1 receptor with Rimonabant, the improvement in LID could be due to the changes in TRPV1 receptor, which, as CB1 is localized in striatum. These and other data led to conclude that the blockade of CB-1 and TRPV1 by ECBs has opposite effects on LID. Hence, the data reported here are somehow in agreement with the results of these authors, since CB1 and TRPV1 receptors were blocked by different paths, however further analysis are needed in order to understand why the co-administration with L-DOPA reduced the severity of AIMS. On the other hand, the fact that Rimonabant in our study demonstrates a reduction in the severity of AIMS is in agreement with the data reported by Fernández-Espejo et al. [36], who found that the systemic administration of cannabinoid CB1 Rimonabant antagonist exerted antiparkinsonian effects in animals with very severe SNc degeneration. In their group of rats, nigral TH+ cell loss was higher than 95% (an analogue of last stage of human PD), and turning, akinesia, sensorimotor neglect and right forepaw use were significantly ameliorated. The mfb 6-OHDA lesion used here produces a severe SNc degeneration, corroborated by apomorphine-induced circling behavior [106] and
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TH+ cell count (See Figs. 4 and 5). According to Fernández-Espejo et al. [36], previous contradictory results after CB1 antagonists in parkinsonian rats and monkeys could be attributable to the differential efficacy of these ligands depending on the level of nigral degeneration. In support of this hypothesis, the reserpine rat model of PD is known to induce a strong DA depletion with a rapid development of striatal DAergic supersensitivity (within 12–24 h after reserpine) [104]. Cannabinoid CB1 antagonists are effective in ameliorating immobility in this model [27]. However, cannabinoid CB1 antagonists have revealed not to be effective in long-term MPTP-treated parkinsonian monkeys [77]. Other authors have shown that most severely injured parkinsonian animals subjected to chronic MPTP regimen present 70–80% nigral TH+ cell loss and >95% of striatal DA depletion [29]. According to the present study, the percentage surviving population of nigral TH+ neurons is a critical factor regarding the antiparkinsonian nature of cannabinoid CB1 antagonist, and its oral administration appears to exert functional effects when nigral loss of DAergic neurons is more than 95%. Therefore, since this antagonist only improved motor inhibition in 6-OHDA-lesioned rats with considerably DAergic degeneration (>95%), it was proposed that low to moderate doses of Rimonabant might only be effective at the advanced phases of PD characterized by severe denervation of the striatum. This observation might serve as a basis to develop antiparkinsonism agents in a stage of the disease when classic DAergic therapy generally fails [44].

On the other hand, Maccarrone et al. [70] demonstrate that in a rat model of PD induced by unilateral nigral lesion with 6-OHDA, the striatal levels of the ECB AEA were increased, while the activity of its membrane transporter (AMT) and FAAH were decreased, these effects were completely reversed by chronic L-DOPA treatment; for that reason it is possible that treatment with L-DOPA is effective at the beginning of the disease. It has been reported that corticostrial glutamatergic transmission is enhanced following DA denervation [11, 18, 52]. This effect also reflects the loss of the D2 receptor-mediated control of corticostrial transmission [19]. Interestingly, D2 and CB1 receptors share the same signal transduction pathway and cooperate closely in the negative regulation of striatal excitatory transmission [77]. Thus, the finding that endogenous levels of AEA are higher in parkinsonian rats may reflect a compensatory mechanism that is trying to control the cortical glutamatergic drive to the striatum. However, this mechanism seems not to be sufficient in chronic L-DOPA-treated rats, since spontaneous excitatory activity, manifested as AIMs, is still higher in these animals. In keeping with this hypothesis, it has been shown that chronic L-DOPA increase CB1 mRNA levels in the striatum of rats unilaterally lesioned with 6-OHDA [114], moreover, Ferrer et al. [40] demonstrate that L-DOPA selectively elevates AEA in different areas of the basal ganglia of normal rats via activation of dopamine D1/D2 receptors. This elevation was prevented by pharmacological blockade of either D1- or D2-like receptors in striatum and Globus Pallidus, and by D1 antagonists only in SNC. The authors hypothesize that this increase represents a negative feedback to enhanced DAergic transmission following L-
DOPA administration. Interestingly, in our experiment the pharmacological blockade of the CB1 or TRPV1 receptors in combination with L-DOPA notably attenuates AIM severity. In regard to capsazepine protection, it has been shown that the administration of the endocannabinoid AEA to rats produces hypokinesia in parallel to a decrease in the activity of nigrostriatal DAergic neurons [22], this effect is also able to activate TRPV1 receptors, and that these receptors are located on nigrostriatal DAergic neurons, suggest that the activation of vanilloid-like receptors rather than CB1 receptors might be responsible of AEA-induced hypokinesia and decreased nigrostriatal dopaminergic activity. These effects were completely reversed by the TRPV1 receptor antagonist capsazepine, thus indicating a role of these receptors in mediating hypokinetic effects of AEA. In vitro studies, using perfused striatal fragments, support this vanilloid-like receptor-mediated direct action, which would not be available for classic cannabinoid agonists. These observations reinforce the notion that the blockade of TRPV1 receptors in the basal ganglia circuitry plays an important role in the control of movement and, complementarily to CB1 receptors, represent another target susceptible of analysis for a potential application in motor disorders.

Cytological alterations.
Dendritic spine counting showed that 6-OHDA and L-DOPA only-treated groups exhibit significant dendritic spine loss in both striata analyzed, and severe TH-positive cell loss in both, ipsilateral and contralateral SNc; it seems that the contralateral side is damaged after DA depletion but in less proportion than the ipsilateral one [3, 4]. Yang et al. [113] argue that changes that occur in the ipsilateral SN affect the contralateral side because their electrophysiological data have shown that the SNc from the contralateral brain side influences nigrostriatal DA cell activity. Moreover, Fass and Butcher [34] and Emsley et al. [32] have reported that nigrostriatal projection is primarily ipsilateral, but also comprises a small contralateral component. Our results also show that both antagonists co-administrated with L-DOPA prevented 6-OHDA-induced spine loss, and since L-DOPA-treatment alone did not avoid this loss, it is possible that the prevention of dendritic spine loss is crucial to the antidyskinetic effects [96] of Rimonabant or capsazepine treatments. It has been demonstrated that profound plastic changes affect the striatal medium size spiny neurons during the progressive loss of DA input [2, 3, 59, 84]. Hence, we hypothesized that the loss of spines and the alterations in the synaptic connectivity observed after 6-OHDA lesion or L-DOPA treatment, might participate in the development of adverse motor events related to L-DOPA therapy [98], because it would alter information flow through the striatum and rest of the basal ganglia nuclei, and since both antagonists prevent the striatal synaptic alterations, it is possible that CB1 or TRPV1 blockade actively participate in the preservation of the striatal synaptic connectivity. Indeed, the excitability of DA receptors is elevated after DA depletion [72, 97]. This adaptation of excitability in parallel with the loss of connectivity could be intimately linked to the development of dyskinesias and might be related to ECB
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system, since El-Banoua et al. [31] demonstrate that CB1 antagonism reduced motor asymmetry in parkinsonian rats after injections into striatum, globus pallidus, and to a lesser extent, subthalamic nucleus. At the level of dorsal striatum, Rimonabant effects were mediated through an opposite modulation of D1 and D2 DA receptor function. Likewise, Martin et al. [74] found that the ECB system is a relevant negative modulator of dopamine D1 and D2 receptor-mediated behaviors through its actions on striatal neurons co-expressing DA and cannabinoid receptors. Moreover, it is possible that the antidyskinetic effect of capsazepine is related to the fact that TRPV1 receptors co-localize with CB1 receptors [20, 27], suggesting a close functional interaction between these receptors.

On the other hand, we found a less severe but significant loss of TH-positive cells in both, contralateral and ipsilateral SNc in the groups co-treated with L-DOPA and antagonists, however this loss was not enough to alter the dendritic spine density in the striatal medium size spiny neurons; the possible explanation is that capsazepine seems to promote DA liberation in striatal terminals [22] and Rimonabant modulates glutamate release over medium size spiny neurons [77], both events protect and/or enhance dendritic spine density.

In this way, it is possible that TH-positive cell death prevention was caused by the antagonism of TRPV1 receptors, which impedes increased calcium influx [83], effect, which might account for the increase of striatal glutamate release by the agonist capsaicin.

There are some reports that cannabinoid agonists may offer neuroprotection in PD [38]. This has been studied in rats with hemiparkinsonism generated by unilateral injection of the neurotoxin 6-OHDA [46, 67]. D9-THC and cannabidiol were the first cannabinoids shown to be capable of attenuating the damage to nigrostriatal DAergic neurons caused by this neurotoxin [67]. Neuroprotective effects of cannabinoids blocked by CB1 receptor antagonists/inverse agonists such as Rimonabant have also been found in other in vivo models of neuronal injury [42], such as, trauma [86] and multiple sclerosis [6]. Moreover, Rimonabant has been used in many studies to assess whether neuroprotective effects of agonists or indirectly acting compounds are mediated via this receptor [42] in models of cerebral ischemia [8, 91, 115], trauma [30], PD [108], and neuronal damage induced by NMDA [54]. Thus, we assume that, since AEA is elevated after DA denervation [70, 109] and in PD patients [93], and that increase stimulate the overproduction of glutamate leading to cell death [19, 52], the antagonistic effect by Rimonabant impedes AEA over activation preventing in part DAergic cell death. Besides, Rinaldi-Carmona et al. [94] show that Rimonabant is a functional antagonist of the brain CB1 receptor with a good oral bioavailability and a long duration of action.

The fact that both antagonists have been effective, although it has been proposed to have opposite effects, lies mainly in the CB1 or TRPV1 receptors status, and also in the severity of DA depletion. As we mentioned above, activation of DA receptors is accompanied by release of AEA throughout the basal ganglia and is
disrupted after lesioning the nigrostriatal pathway, thus the blockade of TRPV1 receptors by capsazepine promotes the release of DA and in consequence improves neuronal survival preventing motor alterations. In regard to Rimonabant, we have mentioned earlier that ECB transmission is augmented after DA depletion suggesting that the blockade of cannabinoid CB1 receptors might be helpful in reducing motor disturbances. In this regard, it has been demonstrated that Rimonabant improved motor inhibition in 6-OHDA-lesioned rats with extremely high degeneration of DAergic neurons (as in our case and in the case of patients with advanced disease). Moreover, it has been proposed that local decreases and increases of ECB tone occur in the striatum of animals and humans with impaired nigrostriatal DA signaling causing different effects on glutamatergic inputs from subthalamic nucleus to GABAergic outputs on the Globus Pallidus external segment and substantia nigra reticulata. Decreases (possibly due to decreased D2 signaling) might occur in glutamatergic neurons impinging on indirect pathway, whereas increases (possibly due to decreased D1 signaling) might occur in glutamatergic neurons affecting the direct pathway. This would result in stimulation or reduction of GABAergic outputs in the two pathways, respectively, thereby causing enhanced GABAergic activity from both the internal layer of the globus pallidus and the substantia nigra reticulata onto thalamic (and hence cortical) outputs, with subsequent locomotor impairments [9]. Therefore, CB1 receptor blockade by Rimonabant could regulate GABAergic transmission to thalamus enhancing glutamatergic transmission to cerebral cortex, preventing the motor alterations.

In summary, our results demonstrate that oral co-administration with L-DOPA/Rimonabant or L-DOPA/capsazepine results in an evident improvement of the AIMS severity, and that improvement correlated with enhanced DAergic cell survival and in consequence, to a well-preserved striatum sinaptology. Advances in defining the role of ECBs in both normal basal ganglia function and in the pathophysiology of PD and LID provide basis for ECB-targeted therapy for these and other movement disorders. However, experimental evidence indicates that the effects of CB1 or TRPV1 receptor agonists or antagonists in the basal ganglia circuits are site-specific and probably dose-dependent. Recent studies in models of PD also indicate the effects of these drugs depend on the severity of DA denervation. Given these multiple variables, the results of clinical trials assessing the efficacy of ECB receptor agonists or antagonists in PD and LID still need to be interpreted with caution. However, the potential therapeutic value of these drugs, not only as adjunctive but also as potential neuroprotective therapy, warrant further studies to better define their use in PD and other movement disorders.

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