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Beneficial effects of the phytocannabinoid Δ^9-THCV in L-DOPA-induced dyskinesia in Parkinson's disease

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ABSTRACT

The antioxidant and CB_2 receptor agonist properties of Δ^9-tetrahydrocannabivarin (Δ^9-THCV) afforded neuroprotection in experimental Parkinson's disease (PD), whereas its CB_1 receptor antagonist profile at doses lower than 5 mg/kg caused anti-hypokinetic effects. In the present study, we investigated the anti-dyskinetic potential of Δ^9-THCV (administered i.p. at 2 mg/kg for two weeks), which had not been investigated before. This objective was investigated after inducing dyskinesia by repeated administration of L-DOPA (i.p. at 10 mg/kg) in a genetic model of dopaminergic deficiency, $Pitx3^{ak}$ mutant mice, which serves as a useful model for testing anti-dyskinetic agents. The daily treatment of these mice with L-DOPA for two weeks progressively increased the time spent in abnormal involuntary movements (AIMs) and elevated their horizontal and vertical activities (as measured in a computer-aided actimeter), signs that reflected the dyskinetic state of these mice. Interestingly, when combined with L-DOPA from the first injection, Δ^9-THCV delayed the appearance of all these signs and decreased their intensity, with a reduction in the levels of FosB protein and the histone pAcH3 (measured by immunohistochemistry), which had previously been found to be elevated in the basal ganglia in L-DOPA-induced dyskinesia. In addition to the anti-dyskinetic effects of Δ^9-THCV when administered at the onset of L-DOPA treatment, Δ^9-THCV was also effective in attenuating the intensity of dyskinesia when administered for three consecutive days once these signs were already present (two weeks after the onset of L-DOPA treatment). In summary, our data support the anti-dyskinetic potential of Δ^9-THCV, both to delay the occurrence and to attenuate the magnitude of dyskinetic signs. Although further studies are clearly required to determine the clinical significance of these data in humans, the results nevertheless situate Δ^9-THCV in a promising position for developing a cannabinoid-based therapy for patients with PD.

1. Introduction

PD is a progressive neurodegenerative disorder whose etiology has been associated with environmental insults, genetic susceptibility, or interactions between both causes (Schapira and Jenner, 2011). The major clinical symptoms in PD are tremor, bradykinesia, postural instability and rigidity (Kim et al., 2018), symptoms that result from the severe dopaminergic denervation of the striatum caused by the progressive death of dopaminergic neurons of the *substantia nigra pars compacta* (Pavón et al., 2006; Sauerbier et al., 2016). Major symptoms in PD (*e.g.* bradykinesia) can be attenuated with dopaminergic replacement therapy using the dopamine precursor L-DOPA (Pezzoli and Zini, 2010). However, this therapy does not work in all PD patients and when used for more than 5–10 years, it loses efficacy and provokes an irreversible dyskinetic state characterized by the appearance of abnormal involuntary movements (Espay et al., 2018). Therefore, the

Abbreviations: AIMs, abnormal involuntary movements; pAcH3, antiphospho(Ser10)-acetyl(Lys14)-hystone 3; AUC, area under the curve; CBD, cannabidiol; CB_1 receptor, cannabinoid receptor type-1; CB_2 receptor, cannabinoid receptor type-2; FAAH, Fatty acid amide hydrolase; 6-OHDA, 6-hydroxydopamine; LPS, lipopolysaccharide; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MAGL, Monoacylglycerol lipase; PD, Parkinson's disease; PPAR, peroxisome proliferator-activated receptor; Δ^9-THC, Δ^9-tetrahydrocannabinol; Δ^9-THCV, Δ^9-tetrahydrocannabivarin

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search for novel symptomatic therapies that are devoid of pro-dyskinetic side effects or able to delay/reduce these signs, in addition to delaying the progression of nigrostriatal damage in PD, remains a major challenge in PD therapy (Kulisevsky et al., 2018).

Cannabinoid-based compounds have been recently proposed as promising therapies in PD given their potential as symptom-alleviating and disease-modifying agents (Fernández-Ruiz, 2009; González-Aparicio and Moratalla, 2014; Fernández-Ruiz et al., 2015; Aymerich et al., 2018; Antonazzo et al., 2019; Cristino et al., 2020; Junior et al., 2020). As regards to the first of these two options, the blockade of the CB_1 receptor, which is highly abundant in basal ganglia structures, may be effective in reducing the motor inhibition typical of PD patients, which is concordant with the overactivity of the cannabinoid system observed in PD patients and animal models of this disease (reviewed in Fernández-Ruiz, 2009). However, the preclinical studies conducted so far have demonstrated that the efficacy of CB_1 receptor blockade was restricted to specific circumstances, *e.g.* the use of low doses, strong nigral damage (Fernández-Espejo et al., 2005; González et al., 2006; Kelsey et al., 2009), conditions that were not reproduced in the only clinical trial conducted so far with a CB_1 receptor blocker, which included a population of patients that were all good-responders to L-DOPA (Mesnage et al., 2004). Therefore, this potential therapeutic strategy merits further clinical investigation, this time with PD patients that respond poorly to L-DOPA (approximately 15–20% of patients are poor responders to L-DOPA and it appears that, in general, they may correspond to those having tremor as the key symptom rather than rigidity and bradykinesia (Mohl et al., 2017)).

Some cannabinoids have been reported to protect nigral neurons from death caused by different insults in various experimental models of PD (reviewed in Fernández-Ruiz, 2009; Fernández-Ruiz et al., 2015; Aymerich et al., 2018; Antonazzo et al., 2019; Cristino et al., 2020; Junior et al., 2020). These include the phytocannabinoids, Δ^9-THC and CBD, the synthetic cannabinoid receptor agonist CP55,940 and the anandamide analog AM404 (reviewed in Fernández-Ruiz, 2009; Fernández-Ruiz et al., 2015; Aymerich et al., 2018). *A priori* these compounds acted through antioxidant mechanisms that seem to be independent of CB_1 or CB_2, although compounds also targeting the CB_2 receptor afforded neuroprotection in MPTP- (Price et al., 2009; Chung et al., 2016), LPS- (García et al., 2011; Gómez-Gálvez et al., 2016) or rotenone-lesioned (Javed et al., 2016) mice, with controversial results in 6-OHDA-lesioned rodents (García-Arencibia et al., 2007; Ternianov et al., 2012). The benefits obtained with CB_2 agonists appeared to depend predominantly on the activation of receptors located in activated astrocytes and/or reactive microglial cells, which would result to be upregulated in the pathology in an attempt to limit the generation of proinflammatory factors (García et al., 2011; Concannon et al., 2015, 2016; Gómez-Gálvez et al., 2016; Navarrete et al., 2018). However, a contribution of CB_2 receptors located in a few neuronal subpopulations, *e.g.* nigrostriatal neurons (García et al., 2015), pallidothalamic neurons (Lanciego et al., 2011), in the basal ganglia cannot be ruled out. In addition, cannabinoids that activate the PPAR nuclear receptors, in particular at the PPAR-γ type, were also neuroprotective in LPS-lesioned mice (García et al., 2018), and those targeting GPR55 may also be beneficial (Celorrio et al., 2017). By contrast, selectively activating the CB_1 receptor, which may elicit ataxia as an adverse effect and/or aggravate major parkinsonian symptoms (*e.g.* bradykinesia), given the hypokinetic effects associated with the activation of this receptor (Fernández-Ruiz, 2009), has been found not to protect against 6-OHDA-induced damage in pharmacological studies (García-Arencibia et al., 2007). However, CB_1 receptor-deficient mice display an increased vulnerability to 6-OHDA lesions (Pérez-Rial et al., 2011), which indicates certain neuroprotective potential exerted by this receptor type. Therefore, these previous data provide good evidence that a cannabinoid having antioxidant properties and the ability to activate CB_2 and PPAR-γ receptors and/or to target GPR55, but to also block CB_1 receptors, might serve to alleviate parkinsonian symptoms and to arrest/delay neurodegeneration in this disease (see Fernández-Ruiz, 2009; Fernández-Ruiz et al., 2015; Aymerich et al., 2018; Antonazzo et al., 2019; Cristino et al., 2020; Junior et al., 2020, for review).

One phytocannabinoid with such a pharmacological profile is Δ^9-THCV. It is antioxidant and has been found to produce signs of CB_1 receptor antagonism (when used at doses lower than 3 mg/kg (Pertwee, 2008)), but also CB_2 receptor activation with significant potency (García et al., 2011). It also appears to have certain agonist activity at the GPR55 (Morales et al., 2017). Using different experimental models of PD, we have demonstrated that Δ^9-THCV alleviates motor inhibition in 6-OHDA-lesioned rodents by blocking CB_1 receptors at low doses, with this effect being similar to that of the classic CB_1 receptor antagonist/inverse agonist, rimonabant (García et al., 2011). It was also able to preserve nigral neurons against different degenerative stimuli in 6-OHDA- and LPS-lesioned mice due to its antioxidant properties and CB_2 agonist activity (García et al., 2011). Its effects in 6-OHDA-lesioned mice were equivalent to those observed with the antioxidant phytocannabinoid CBD (Lastres-Becker et al., 2005; García-Arencibia et al., 2007), whereas those found in LPS-lesioned mice were similar to those found with HU-308, a selective CB_2 receptor agonist (Gómez-Gálvez et al., 2016).

In the present study, we have investigated for the first time whether Δ^9-THCV, used at a low dose, is also anti-dyskinetic, a relevant property for any antiparkinsonian agent. Previous studies implicated modulation of endocannabinoid signaling, in particular targeting the CB_1 receptor, in delaying/reducing L-DOPA-induced dyskinesia (reviewed in Fernández-Ruiz, 2009; Fernández-Ruiz et al., 2015; Aymerich et al., 2018). For example, CB_1 receptor-deficient mice showed less severe dyskinetic signs when lesioned with 6-OHDA and treated with L-DOPA in comparison with wild-type animals (Pérez-Rial et al., 2011). This supports the hypothesis that pharmacological blockade of this receptor may be beneficial for L-DOPA-induced dyskinesia, which has already been investigated in some studies using experimental models (Segovia et al., 2003; Cao et al., 2007; Gutiérrez-Valdez et al., 2013). Benefits were also found after activation of the CB_1 receptor (Segovia et al., 2003; Morgese et al., 2007; Martinez et al., 2012), demonstrating the extreme complexity of the role exerted by this modulatory system in the basal ganglia. In any case, this potential has not been corroborated yet in clinical studies (Carroll et al., 2004). A recent study has added the CB_2 receptor to those endocannabinoid targets susceptible to serve for treating L-DOPA-induced dyskinesia (Rentsch et al., 2020). The current study was designed to explore the benefits of Δ^9-THCV against L-DOPA-induced dyskinesia elicited in a genetic model of dopaminergic deficiency, $Pitx3^{ak}$ mutant mice, which has been widely used to test anti-dyskinetic agents (Hwang et al., 2005; Solís et al., 2015; Suárez et al., 2018). These mice are characterized by hypomorphic expression of the transcription factor gene Pitx3 due to a spontaneous mutation in the aphakia locus affecting the lens-brain-specific promoter, and leaving intact the muscle-specific promoter (Luk et al., 2013; Del Río-Martín et al., 2019). The hypomorphic Pitx3 expression severely affects differentiation of dopamine neurons with a dramatic reduction in number of neurons in the *substantia nigra pars compacta* and strong lack of dopamine in the striatum (Alberquilla et al., 2020). Our study investigated first whether the treatment with Δ^9-THCV administered daily in parallel to L-DOPA was associated with a delay in the appearance of dyskinetic movements, and also whether Δ^9-THCV was able to reduce dyskinetic signs when administered once the L-DOPA-induced dyskinesia was already established. In addition, given that the experimental model used is the first time that was investigated in relation with cannabinoids, our study also included an analysis of the status of major endocannabinoid elements in wildtype and $Pitx3^{ak}$ mutant mice at the age that animals were used for pharmacological treatments.

2. Materials and methods

2.1. Animals and treatments

2.1.1. Animals

Pitx3^{ak} mutant male mice (Nunes et al., 2003; Suárez et al., 2016) and their wildtype male littermates, bred in the animal facilities of the Cajal Institute, were genotyped using PCR amplification analysis of tail-tip DNA extracts. Mice were housed under a 12 h dark/light cycle with free access to food and water. They were used for experimental purposes when they were 4–6 months-old (weighing 24–30 g). Animal care and experimental procedures were conducted according to European rules (directive 2010/63/EU) and ARRIVE guidelines, and were approved by the Ethics Committee of the "Consejo Superior de Investigaciones Científicas (CSIC)". All efforts were made to minimize the number of animals used in this study.

2.1.2. L-DOPA and Δ^9-THCV treatment

In the first experiment, animals were treated with an i.p. injection of 10 mg/kg of benserazide hydrochloride (Sigma-Aldrich, Madrid, Spain) followed 20 min later by a second i.p. injection of 10 mg/kg of L-DOPA methyl ester (Sigma-Aldrich, Madrid, Spain). Separate groups of animals received equivalent injections with vehicle (saline) to be used as controls. Both L-DOPA- and vehicle-treated mice received, 10 min after the second injection, a third i.p. injection with the phytocannabinoid Δ^9-THCV (2 mg/kg; GW Research Ltd., Cambridge, UK) or vehicle (Tween 80-saline). The doses for the three compounds were chosen following previous studies (García et al., 2011; Solís et al., 2015), in particular in the case of the dose of 2 mg/kg for Δ^9-THCV, the selection was based on preliminary data to confirm its activity as a CB_1 receptor antagonist and a CB_2 receptor agonist (García et al., 2011) following previous studies (reviewed in Pertwee, 2008). The same treatment schedule was repeated daily for two weeks. In the second experiment, benserazide and L-DOPA (and their corresponding vehicles) were administered to mice following the same schedule as the previous experiment, but Δ^9-THCV, at 2 mg/kg, was given for the first time after two weeks of daily benserazide/L-DOPA treatment, with the administration of the three compounds (and vehicles) extending for at least 3 days.

2.2. Behavioral measurements

2.2.1. Abnormal involuntary movements

AIMs were assessed 30 and 60 min after L-DOPA administration. Previous studies (Pavón et al., 2006; Ruiz-DeDiego et al., 2015) have demonstrated that the incidence and intensity of AIMs are maximal at 30 and 60 min following L-DOPA administration. Dyskinesia was scored off-line by an assessor who was blind to treatment group allocation based on video footage (Ding et al., 2007; Solís et al., 2015; Ruiz-DeDiego et al., 2018; García-Montes et al., 2019). Ratings were assessed for four minutes at each time point. Total dyskinesia was rated by adding the duration in seconds of all three-paw and four-paw dyskinetic bouts, as described previously (Suárez et al., 2016). Additionally, on odd days, individual mice were placed in actimeters and assessed for motor activity following drug administration. On Day 14, AIMs were evaluated every 20 min for 160 min after administering L-DOPA to determine the extinction of this response (Solis et al., 2015).

2.2.2. Locomotor activity

Horizontal and vertical activities were recorded, as described previously (Centonze et al., 2003; Granado et al., 2008), using a multicage activity meter system (Columbus Instruments, Columbus, OH, USA) consisting of a set of 8 individual cages measuring 20 × 20 × 28 cm. Animals were introduced in the actimeter 60 min after L-DOPA injection and were assessed for 30 min. Horizontal movement was detected by 2 arrays of 16 infrared beams, whereas a third array positioned 4 cm above the floor detected vertical movement. The software can differentiate between repetitive interruptions of the same photobeam and interruptions of adjacent photobeams. The latter measure was used as an index of ambulatory activity.

2.3. Sampling and tissue preparation

Following behavioral analysis, animals were euthanized 1 h after the last injection of L-DOPA with an overdose of pentobarbital (Laboratorios Normon, Madrid, Spain), injected intracardially with 0.5 mL of 1% heparin (Rovi, Madrid, Spain) and then perfused with 10 mL of saline and 50 mL of 4% paraformaldehyde in phosphate buffer (pH 7.4). The brains were post-fixed for 24 h and were then transferred to a solution of 0.1 M phosphate buffer containing 0.02% sodium azide for storage at 4 °C. To obtain regular blocks, brains were further immersed in 3% agarose and cut in coronal sections (30 μm thick) using a vibratome (Leica, Wetzlar, Germany), as described previously (Granado et al., 2010). As a supplementary data, naïve wildtype and Pitx3^{ak} mutant mice (4–6 months-old; 24–30 g) were euthanized and their brains removed and dissected to obtain the striatum and the substantia nigra. Tissues were rapidly frozen and stored at −80 °C up to be used for qPCR analysis of different endocannabinoid genes (see details in supplementary Fig. 1).

2.4. Immunohistochemistry

Immunostaining was carried out by researchers who were blind to the identities of the treatment groups in free-floating sections using a standard avidin-biotin immunohistochemical protocol (Suárez et al., 2016, 2018) with the following rabbit antisera: (i) FosB (1:7500, Santa Cruz Biotechnology, Santa Cruz, California), and (ii) pAcH3 (1:500; Upstate, Cell Signaling Solutions, Lake Placid, New York). Briefly, after incubation with primary antibody (overnight), the sections were washed and incubated with biotinylated secondary anti-rabbit antibody (1:500) (Vector Laboratories) for 1 h at room temperature. After washing, the sections were incubated with streptavidin (Zymed, San Francisco, CA, USA) for 1 h and antibody staining was developed using DAB (Sigma-Aldrich, Madrid, Spain). After developing the reaction, stained sections were mounted, dried, dehydrated, and coverslipped with Permount mounting medium (Fisher Chemicals, Fair Lawn, NJ, USA).

For quantification of FosB and pAcH3 immunoreactivity in the completely denervated area, we used the ImageJ analysis system (Schneider et al., 2012) as previously shown (Ares-Santos et al., 2012; Espadas et al., 2012; Solís et al., 2015). For all sides, immunostaining intensity and number of immunolabeled nuclei were determined using five serial rostrocaudal sections per animal and two counting frames (dorsal and lateral) per section (0.091 mm^2 each frame). Images were digitized with Leica microscope under 40× lens. Before counting, images were thresholded at a standardized gray-scale level. The data are presented as number of stained nuclei per mm^2 (mean ± standard error of the mean) in the lesioned striatum.

2.5. Real time qRT-PCR analysis

Total RNA was extracted from striatal and nigral samples using either SurePrep RNA/Protein Purification Kit (Fisher Bioreagents, Madrid, Spain) or RNeasy Lipid Tissue Minikit (Qiagen, Izasa, Madrid, Spain). The total amount of RNA extracted was quantitated by spectrometry at 260 nm and its purity was evaluated by the ratio between the absorbance values at 260 and 280 nm. After genomic DNA was removed (to eliminate DNA contamination), single-stranded complementary DNA was synthesized from 0.25 μg (or higher) of total RNA using Rneasy Mini Quantitect Reverse Transcription (Qiagen, Izasa, Madrid, Spain) and the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The reaction mixture was kept frozen at −20 °C until

enzymatic amplification. Quantitative real-time PCR assays were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) to quantify mRNA levels for CB_1 receptor (ref. Mm00432621_s1), CB_2 receptor (ref. Mm00438286_m1), FAAH (ref. Mm00515684_m1), and MAGL (ref. Mm00449274_m1), using GAPDH expression (ref. Mm99999915_g1) as an endogenous control gene for normalization. The PCR assay was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the threshold cycle (Ct) was calculated by the instrument's software (7300 Fast System, Applied Biosystems, Foster City, CA, USA). Data were expressed as percentages over the wild-type group.

2.6. Statistical analysis

Data were normally distributed (tested with the Shapiro-Wilk normality test) and were assessed by one-way or two-way (with repeated measures) analysis of variance, as required (see specific test used in the legends to figures), followed by the Bonferroni test, using GraphPad Prism® software (version 5.01; GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Status of several endocannabinoid elements in wildtype and Pitx3^{ak} mutant mice

Our study was initiated with the analysis of the status of the major endocannabinoid elements (CB_1 and CB_2 receptors, and FAAH and MAGL enzymes) in Pitx3^{ak} mutant mice compared to control animals, given that this was the first time that this experimental model was used in cannabinoid research. This was conducted in basal conditions using qPCR, but not after the treatment with L-DOPA and/or Δ^9-THCV, whose tissues were used for other purposes (immunohistochemistry). Our data proved that gene expression for CB_1 and CB_2 receptors and FAAH and MAGL enzymes were similar in the striatum and the substantia nigra of wildtype and Pitx3^{ak} mutant mice, with the only exception of an increase in MAGL found exclusively in the striatum (see Supplementary Fig. 1).

3.2. Development of L-DOPA-induced dyskinesia in Pitx3^{ak} mutant mice

In accordance with previous reports (Ding et al., 2007; Li and Zhou, 2013), the chronic treatment of Pitx3^{ak} mutant mice with L-DOPA resulted in the progressive appearance of AIMs (including front paw, hind paw, three-paw, and four-paw dyskinetic movements; Fig. 1). As a measure of the intensity of dyskinesia, the three- and four-paw dyskinetic movements (Ding et al., 2011) were measured at 30 min (treatment: $F(3,294) = 124.4$, $p < .0001$; time: $F(6,294) = 10.81$, $p < .0001$; Fig. 1A) and 60 min (treatment: $F(3,294) = 73.50$, $p < .0001$; time: $F(6,294) = 5.61$, $p < .0001$; Fig. 1B) after administering L-DOPA. Dyskinetic movements were already evident after the first injection of L-DOPA (treatment: $F(3,88) = 3.01$, $p < .05$), although the posthoc analysis did not reveal any statistical significance compared to vehicle-treated groups (Fig. 1C), but the time spent in AIMs was significantly elevated during the first week of treatment (Fig. 1A,B). After this, the values of the dyskinetic movements remained stable for the rest of the chronic treatment (Fig. 1A,B). On the last treatment day, a time-course was conducted to determine the extinction of dyskinetic movements, which revealed that they were present for at least 2 h after administering L-DOPA (treatment: $F(3,336) = 60.99$, $p < .0001$; time: $F(7,336) = 17.49$, $p < .0001$; Fig. 1D).

The dyskinetic profile shown by Pitx3^{ak} mutant mice after a chronic treatment with L-DOPA also included elevated horizontal (treatment: $F(3,252) = 100.2$, $p < .0001$; time: $F(5,252) = 12.34$, $p < .0001$; Fig. 2A) and vertical (treatment: $F(3,252) = 37.73$, $p < .0001$; time: $F(5,252) = 6.84$, $p < .0001$; Fig. 2B) activities measured in a computer-aided actimeter. The elevation was found after four days of chronic L-DOPA treatment, and it remained during the second week of treatment (Fig. 2A,B).

3.3. Δ^9-THCV delays the appearance of L-DOPA-induced dyskinesia in Pitx3^{ak} mutant mice

The first objective in this study was to evaluate whether Δ^9-THCV could delay the appearance of L-DOPA-induced dyskinetic signs in Pitx3^{ak} mutant mice. Thus, the co-administration of Δ^9-THCV with L-DOPA reduced the duration of AIMs (three/four-paw dyskinetic movements) at 30 min post-L-DOPA administration, except for days 1 and 3 (2-way interaction: $F(18,294) = 3.84$, $p < .0001$; Fig. 1A), a fact also reflected in AUC value for the treatment with L-DOPA alone (859.8 s x days) compared to that calculated for the co-treatment with L-DOPA and Δ^9-THCV (598.7 s x days). This effect was, however, much more modest when analyzed at 60 min after L-DOPA administration (2-way interaction: $F(18,294) = 1.95$, $p < .05$), remaining only as numerical trend towards a decrease but with no statistical differences between L-DOPA (AUC value: 684.5 s x days) and L-DOPA + Δ^9-THCV (AUC value: 551.8 s x days) groups after the posthoc analysis (Fig. 1B). This fact is related more to the reduction in the scores for AIMs at 60 min (compared to 30 min) in the L-DOPA-treated group, rather than to changes in the scores of L-DOPA + Δ^9-THCV group, which at both times appear relatively similar (Fig. 1A,B).

On the last day of treatment, the differences between animals treated with L-DOPA and Δ^9-THCV and those treated with L-DOPA alone remained up to 80 min post-treatment and disappeared afterwards (2-way interaction: $F(21,336) = 6.01$, $p < .0001$; Fig. 1D), but they were simply numerical trends towards a decrease with the posthoc analysis (Fig. 1D). It is important to remark that the treatment of Pitx3^{ak} mutant mice with Δ^9-THCV alone did not induce any dyskinetic movements in any case showing values always similar to vehicle-treated animals at all times (Fig. 1A–D).

The co-administration of Δ^9-THCV with L-DOPA attenuated the elevated horizontal (2-way interaction: $F(15,252) = 5.25$, $p < .0001$; Fig. 2A) and vertical (2-way interaction: $F(15,252) = 2.80$, $p < .0005$; Fig. 2B) activities found in L-DOPA-treated Pitx3^{ak} mutant mice (Fig. 2A,B), in particular at days 8, 10 and 12 for horizontal activity (Fig. 2A) and at day 10 for vertical activity (Fig. 2B). Such beneficial effects were found at 60 min after L-DOPA treatment (Fig. 2A,B), which contrasts with the fact that AIMs were only slightly reduced at that time (Fig. 1B). However, it is necessary to remark that both tests possibly reflect different aspects of motor behavioral anomalies (dyskinetic movements in the first one *versus* these signs and also excess of ambulatory and rearing activities in the multicage activity meter system) elicited by L-DOPA and it may be normal that the timing for these responses may be different. On the other hand, whereas the treatment with Δ^9-THCV alone has no effect on the horizontal activity compared to Pitx3^{ak} mutant mice treated with vehicle (Fig. 2A), the phytocannabinoid administered alone to these mice resulted in a numerical trend towards an elevation in vertical activity, although this elevation was not statistically significant compared to vehicle-treated mice and did not reach the level of animals treated with L-DOPA (Fig. 2B). This effect could be related to the anti-hypokinetic action of Δ^9-THCV described previously (García et al., 2011).

3.4. Δ^9-THCV reduced striatal molecular determinants of L-DOPA treated Pitx3^{ak} mutant mice

Previous studies described an elevated FosB expression induced by L-DOPA treatment in the dopamine-denervated striatum of Pitx3^{ak} mutant mice, which correlates with the appearance of dyskinesia (Pavón et al., 2006; Solís et al., 2015; García-Montes et al., 2019), and a similar elevation in the phosphoacetylation of histone 3 in the striatum (Darmopil et al., 2009). The elevation in both striatal molecular

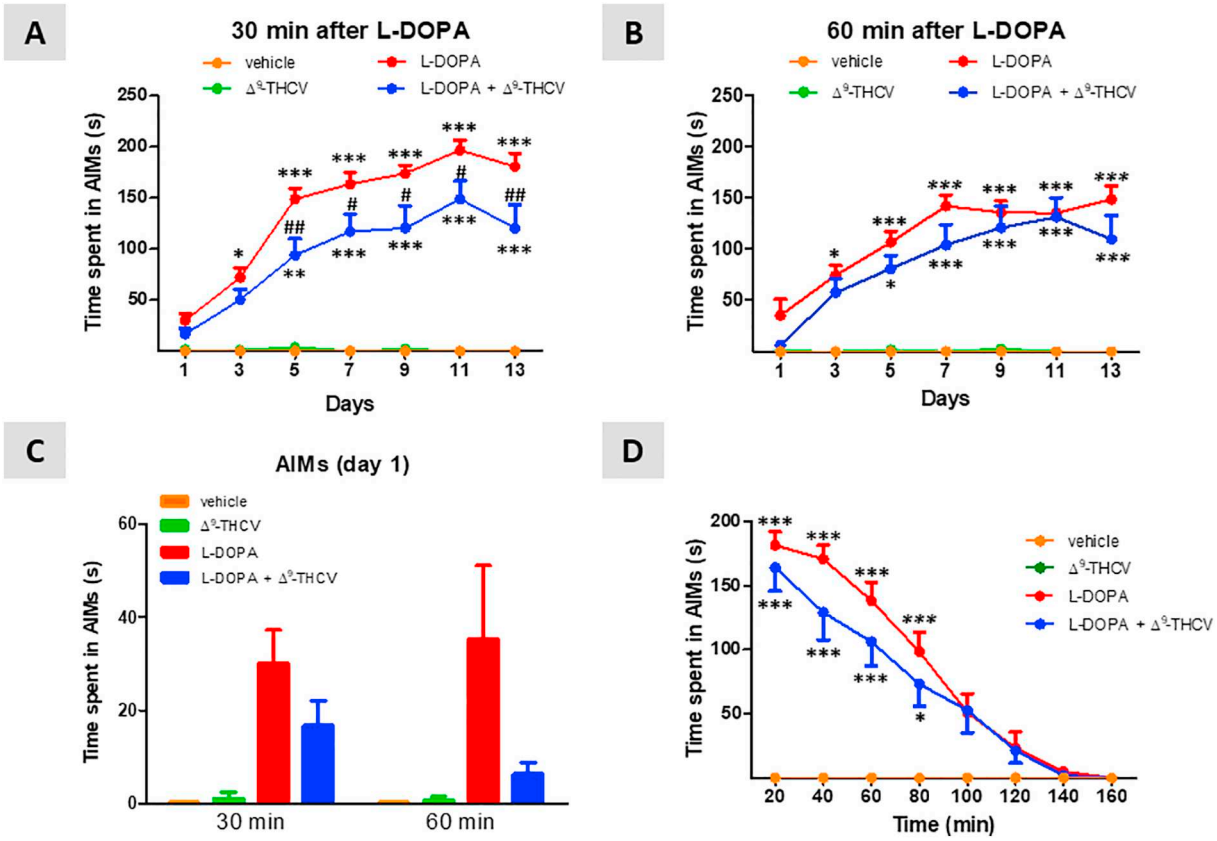

Fig. 1. The effect of Δ^9-THCV in the development of L-DOPA-induced dyskinesia in Pitx3^{ak} mutant mice. Chronic Δ^9-THCV treatment attenuates dyskinesia at 30 min (A) and, to a lesser extent, at 60 min (B) post-L-DOPA. A two-way ANOVA with repeated measures followed by the Bonferroni test showed significant differences at 30 min (treatment: $F(3,294) = 124.4$, $p < .0001$; time: $F(6,294) = 10.81$, $p < .0001$; interaction: $F(18,294) = 3.84$, $p < .0001$) and 60 min (treatment: $F(3,294) = 73.50$, $p < .0001$; time: $F(6,294) = 5.61$, $p < .0001$; interaction: $F(18,294) = 1.95$, $p < .05$). C: The acute co-treatment with Δ^9-THCV and L-DOPA decreases, but not significantly, the dyskinesia score at 30 min and 60 min on the day 1 of treatment, using a two-way ANOVA with repeated measures followed by the Bonferroni test (treatment: $F(3,88) = 3.01$, $p < .05$; time: $F(1,88) = 0.02$, ns; interaction: $F(3,88) = 0.14$, ns). D: The kinetic profile of dyskinetic symptoms was evaluated once every 20 min over 160 min on day 13 of the L-DOPA treatment. A two-way ANOVA with repeated measures followed by the Bonferroni test showed significant differences (treatment: $F(3,336) = 60.99$, $p < .0001$; time: $F(7,336) = 17.49$, $p < .0001$; interaction: $F(21,336) = 6.01$, $p < .0001$). The data are expressed as the mean ± SEM of at least 6 animals *per* group; *$p < .05$, **$p < .01$, ***$p < .005$ *versus* mice treated with vehicle or Δ^9-THCV alone; #$p < .05$, ##$p < .01$ *versus* mice treated with L-DOPA alone.

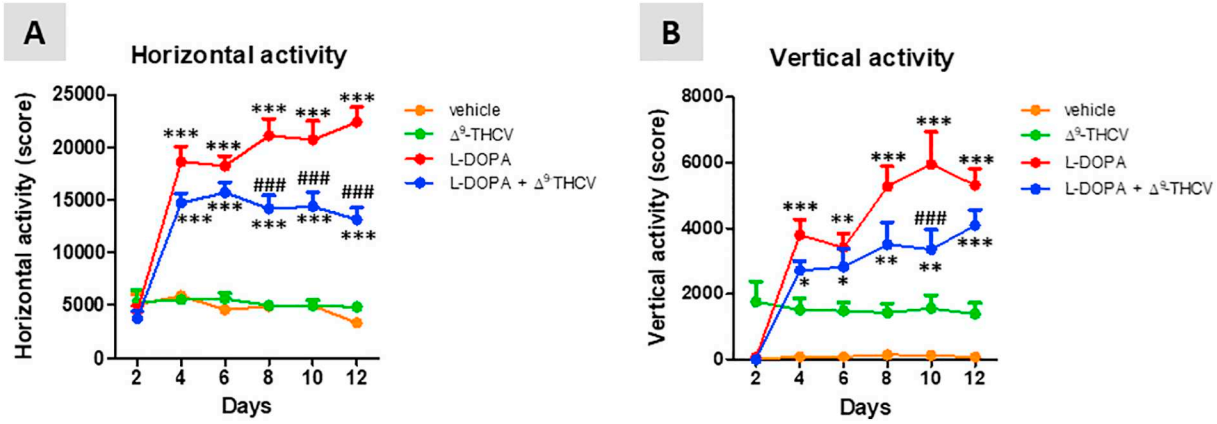

Fig. 2. Chronic Δ^9-THCV treatment decreases motor hyperactivity induced by L-DOPA in Pitx3^{ak} mutant mice. Horizontal (A) and vertical (B) motor activities were measured in a multicage activity meter system 60 min after L-DOPA or vehicle challenge. A two-way ANOVA with repeated measures followed by the Bonferroni test showed significant differences for the horizontal (treatment: $F(3,252) = 100.2$, $p < .0001$; time: $F(5,252) = 12.34$, $p < .0001$; interaction: $F(15,252) = 5.25$, $p < .0001$) and vertical (treatment: $F(3,252) = 37.73$, $p < .0001$; time: $F(5,252) = 6.84$, $p < .0001$; interaction: $F(15,252) = 2.80$, $p < .0005$) activities. The data are expressed as the mean ± SEM of at least 6 animals *per* group; *$p < .05$, **$p < .01$, ***$p < .005$ *versus* mice treated with vehicle or Δ^9-THCV alone; ###$p < .001$ *versus* mice treated with L-DOPA alone.

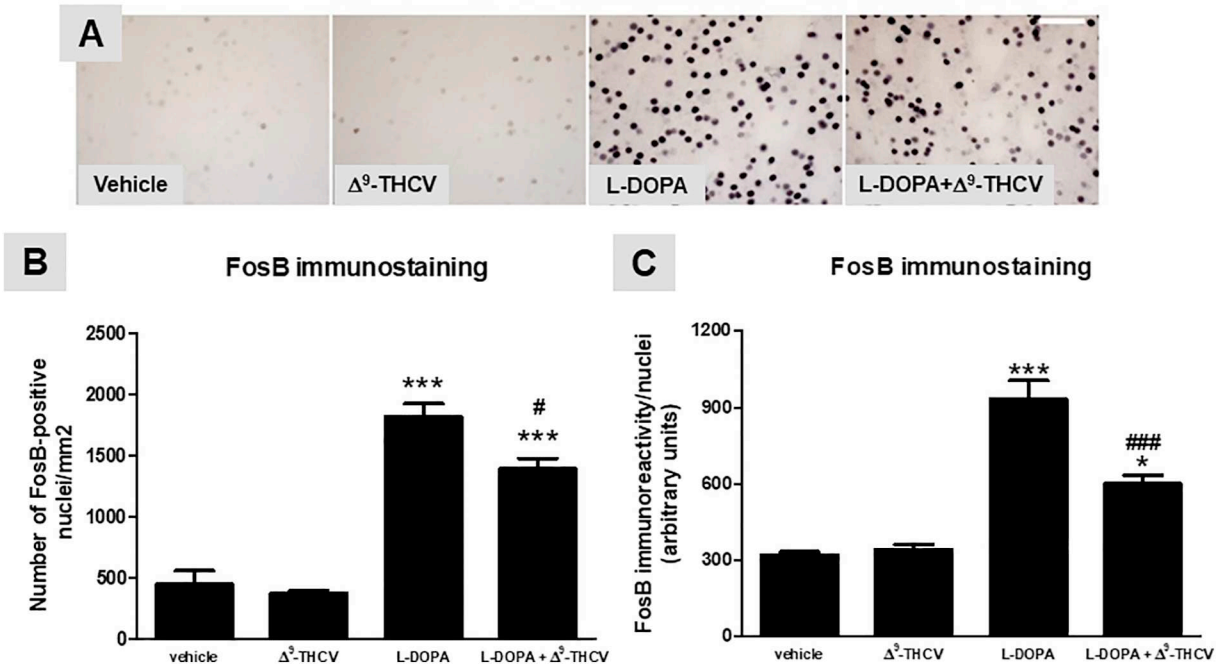

Fig. 3. Δ^9-THCV treatment decreases L-DOPA-induced FosB expression in the striatum of Pitx3^{ak} mutant mice. A: High-power microphotographs of striatal sections from Pitx3^{ak} mutant mice illustrating the effect of chronic Δ^9-THCV treatment on L-DOPA-induced FosB expression. B: Striatal quantification of FosB-positive cells after different treatments. The Δ^9-THCV challenge attenuates the increased expression of FosB-positive cells caused by L-DOPA treatment. Histograms represent the number of FosB nuclei. C: In addition, the Δ^9-THCV treatment attenuates the immunostaining-intensity of FosB positive nuclei. Histograms represent a comparative of immunostaing-intensity in a relative scale. The data are expressed as the mean ± SEM of at least 4 animals *per* group and they were analyzed by one-way ANOVA followed by the Bonferroni test (positive nuclei: $F(3,25) = 32.1$, $p < .0001$; immunoreactivity: $F(3,25) = 17.5$, $p < .0001$). *p < .05, ***p < .005 *versus* mice treated with vehicle or Δ^9-THCV alone; #p < .05, ###p < .005 *versus* mice treated with L-DOPA alone. Scale bar = 50 μm.

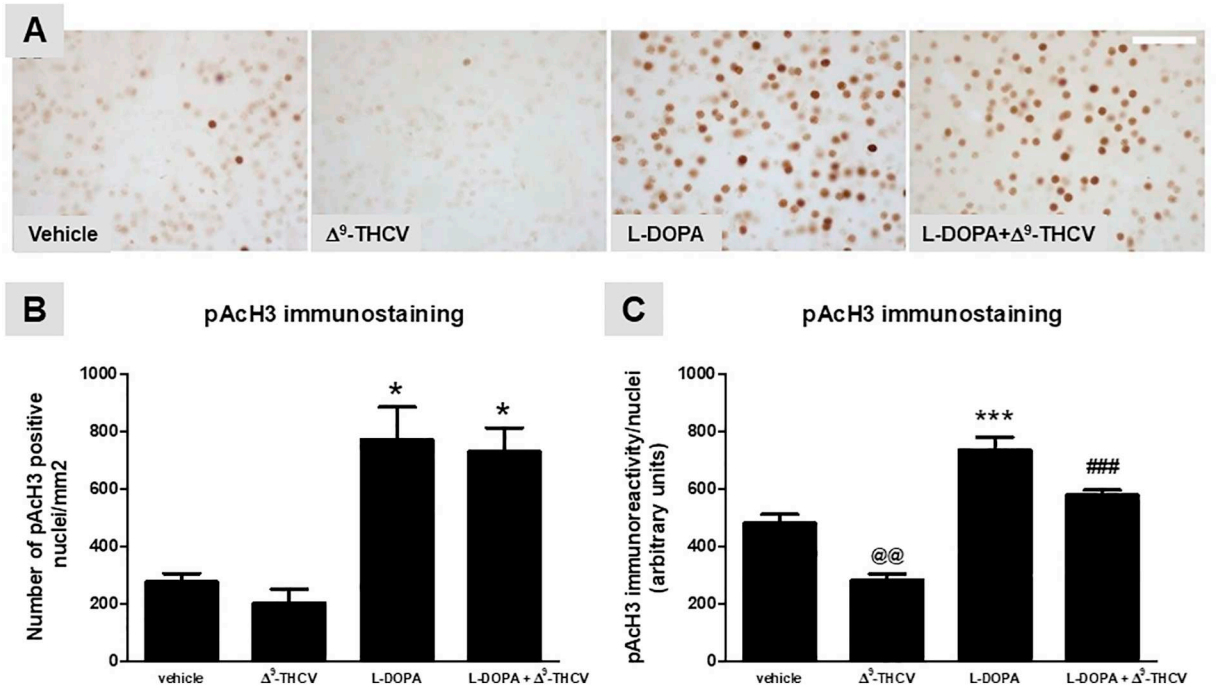

Fig. 4. Treatment with Δ^9-THCV decreases L-DOPA-induced pAcH3 expression in the striatum of Pitx3^{ak} mutant mice. A: High-power microphotographs of the striatal sections of Pitx3^{ak} mutant mice illustrating the effect of chronic Δ^9-THCV treatment on pAcH3 expression induced by L-DOPA. B: Striatal quantification of pAcH3-positive cells after different treatments. The Δ^9-THCV challenge attenuates the increased expression of pAcH3-positive cells caused by L-DOPA treatment. Histograms represent the number of pAcH3 nuclei. C: In addition, the Δ^9-THCV treatment attenuates the immunostaining-intensity of pAcH3 positive nuclei. Histograms represent a comparative of immunostaing-intensity in a relative scale. The data are expressed as the mean ± SEM of at least 4 animals *per* group and they were analyzed by one-way ANOVA followed by the Bonferroni test (positive nuclei: $F(3,24) = 6.62$, $p < .005$; immunoreactivity: $F(3,24) = 26.86$, $p < .0001$). *p < .05, ***p < .005 *versus* mice treated with vehicle or Δ^9-THCV alone; ###p < .005 *versus* mice treated with L-DOPA or Δ^9-THCV alone; @@ p < .01 *versus* mice treated with vehicle alone. Scale bar = 50 μm.

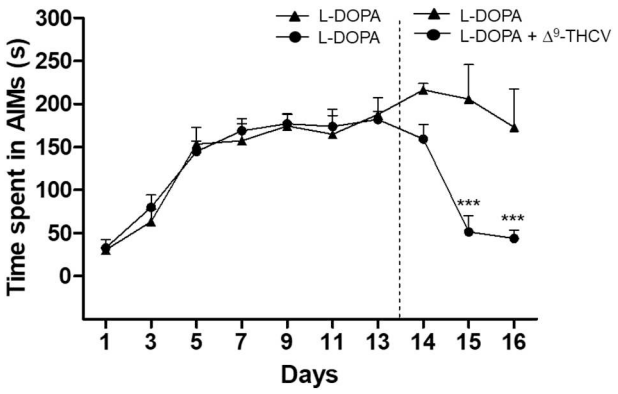

Fig. 5. The effect of Δ^9-THCV on established L-DOPA-induced dyskinesia at the first peak-dose of L-DOPA. Pitx3^{ak} mutant mice received daily injections of L-DOPA for 13 days to establish dyskinetic status. On day 14, 15 and 16, animals received Δ^9-THCV or vehicle 10 min after L-DOPA injection. The Δ^9-THCV treatment significantly reduced dyskinesia at 30 min post-L-DOPA. The data are expressed as the mean ± SEM of at least 6 animals *per* group. Two-way ANOVA followed by the Bonferroni test showed significant differences (treatment: $F(1,166) = 14.17$, $p < .0005$; time: $F(9,166) = 15.75$, $p < .0001$; interaction: $F(9,166) = 5.54$, $p < .0001$). ***$p < .005$ *versus* L-DOPA.

markers has been also observed in the present study using immunostaining in L-DOPA-treated Pitx3^{ak} mutant mice (Figs. 3A and 4A). Interestingly, both responses were significantly attenuated after the co-administration with Δ^9-THCV (Figs. 3A and 4A). In the case of FosB, the Δ^9-THCV-induced reduction affected both the number ($F(3,25) = 32.07$, $p < .0001$; Fig. 3B) and the immunostaining intensity ($F(3,25) = 17.55$, $p < .0001$; Fig. 3C) of FosB-positive cells compared to the chronic L-DOPA-treated mice, whereas the lowering effect in the case of pACH3 was only evident in the intensity of immunostained-nuclei (Fig. 4B,C) and was also seen when Δ^9-THCV was given alone ($F(3,24) = 26.86$, $p < .0001$; Fig. 4C).

3.5. Delayed treatment with Δ^9-THCV also attenuated the magnitude of L-DOPA-induced dyskinesia in Pitx3^{ak} mutant mice

The second objective was to further explore the antidyskinetic potential of Δ^9-THCV by investigating whether, in addition to its capability to delay the appearance of dyskinetic signs, Δ^9-THCV could affect the intensity of dyskinesia when administered once these signs were already present. To this end, Δ^9-THCV treatment commenced two weeks after the onset of L-DOPA treatment. As shown in Fig. 5, animals chronically treated with L-DOPA developed a progressive elevation in AIMs (in general, similar to the data presented in Fig. 1A) up to day 13 (Fig. 5) when they were randomly distributed in two different subgroups, treated with L-DOPA alone or co-treated with L-DOPA and Δ^9-THCV. Mice treated with Δ^9-THCV from day 14 onwards displayed a reduction in the duration of L-DOPA-induced three/four-paw dyskinesia 30 min after the L-DOPA injection, in comparison with the group that received L-DOPA alone, and this persisted for 3 days (treatment: $F(1,166) = 14.17$, $p < .0005$; time: $F(9,166) = 15.75$, $p < .0001$; 2-way interaction: $F(9,166) = 5.54$, $p < .0001$; Fig. 5).

4. Discussion

The gold standard therapy in PD is L-DOPA, which is an intermediate metabolite in dopamine synthesis that easily crosses the blood-brain barrier. L-DOPA is not subjected to the enzymatic limitation that occurs with the natural precursor, L-tyrosine, and thus provides a rapid generation of dopamine in synapses due to its fast decarboxylation in surviving dopaminergic/serotonergic terminals and other striatal neural substrates (Carta et al., 2007; Hadjiconstantinou and Neff, 2008). L-DOPA therapy helps a relatively high percentage of patients with PD, excluding frequently those having tremor as the key symptom (Mohl et al., 2017), but, after 5–10 years of daily use, it causes the appearance of abnormal involuntary movements, so-called dyskinetic movements, as well as a loss of efficacy that represents an important problem for patients (Espay et al., 2018). Efforts are being made in the search for new symptom-alleviating agents that do not generate dyskinetic signs, or in the identification and development of coadjuvants that may be combined with L-DOPA with the objective of using lower (and less dyskinetic) doses of this precursor, or to attenuate/delay the occurrence of side effects associated with normal (and prodyskinetic) doses (Johnston et al., 2019).

Among the different mechanisms and targets investigated regarding pathogenesis and potential new therapies for dyskinesia, one attractive option is the endocannabinoid system and, in particular, the CB_1 receptor (Fernández-Ruiz, 2009; González-Aparicio and Moratalla, 2014; Aymerich et al., 2018). Despite controversy surrounding the data generated to date, interest in these targets remains, with beneficial effects found with both the activation (Segovia et al., 2003; Morgese et al., 2007; Martinez et al., 2012) and the blockade (Segovia et al., 2003; Cao et al., 2007; Gutiérrez-Valdez et al., 2013) of the CB_1 receptor (see details in the Introduction). Our present study is not designed to further investigate the reasons for such controversy, but to determine whether the plant-derived cannabinoid, Δ^9-THCV, whose pharmacological profile appears attractive for PD (it may be neuroprotective due to its antioxidant and CB_2 receptor agonist properties, as well as anti-hypokinetic derived from its CB_1 receptor antagonist profile at doses lower than 5 mg/kg; see García et al., 2011), may also serve as an anti-dyskinetic agent, a property extremely useful for any antiparkinsonian agent. Our study is the first one to investigate Δ^9-THCV for its anti-dyskinetic potential. To achieve this, we used Pitx3^{ak} mutant mice, which are considered a reliable animal model of parkinsonism associated with dopaminergic deficiency, given the role played by the transcription factor Pitx3 in the development of midbrain dopamine neurons (Nunes et al., 2003; Hwang et al., 2003; van den Munckhof et al., 2003). In fact, Pitx3 polymorphisms have been associated with PD (Fuchs et al., 2009). Pitx3^{ak} mutant mice present a selective bilateral dopamine depletion in the nigrostriatal system that is associated with impaired spontaneous locomotor activity showing akinesia (Nunes et al., 2003; van den Munckhof et al., 2003), then serving as a tool to investigate drugs with antiparkinsonian activity (van den Munckhof et al., 2006; Solís et al., 2015). This also includes L-DOPA, for which Pitx3^{ak} mutant mice are highly responders (Hwang et al., 2005), even developing dyskinesias following repeated L-DOPA administration (Iderberg et al., 2012) and reproducing the biochemical, histochemical and synaptic plasticity changes induced by L-DOPA in other models of experimental parkinsonism (Ding et al., 2007, 2011; Espadas et al., 2012; Li and Zhou, 2013; Alberquilla et al., 2020). In our study, the appearance of dyskinetic signs (AIMs, and horizontal and vertical hyperactivity) during the treatment of Pitx3^{ak} mutant mice with L-DOPA was significantly prevented by co-administration with Δ^9-THCV. Our data also indicated that the anti-dyskinetic effect of Δ^9-THCV was likely associated with a reduction in two molecular markers, FosB and pAcH3, that have been found to be exacerbated in the dopamine-denervated striatum after L-DOPA treatment (Pavón et al., 2006; Darmopil et al., 2009; Solís et al., 2015), more particularly in striatal neurons of the direct pathway as we demonstrated by double immunostaining and optogenetic stimulation (Keifman et al., 2019).

It is important to remark that this beneficial effect of Δ^9-THCV was observed when the phytocannabinoid was co-administered with L-DOPA during the whole period of treatment, thus supporting the idea that Δ^9-THCV delays the occurrence of L-DOPA-induced dyskinesia. However, we also wanted to investigate whether Δ^9-THCV also exhibited anti-dyskinetic properties when administered once L-DOPA-induced dyskinesia was already established, and this was again the case. Our data indicated that the intensity of dyskinetic signs was attenuated when Δ^9-THCV was co-administered with L-DOPA for 3 days after

13 days of daily treatment with L-DOPA alone. This additional observation is extremely important to support the anti-dyskinetic profile of Δ^9-THCV that adds to the other therapeutic benefits described for this phytocannabinoid in previous studies (García et al., 2011).

As regards to the mechanism(s) that may explain the anti-dyskinetic effect found for Δ^9-THCV in our study, the first option would be its activity as a CB_1 receptor antagonist at the dose used here. This should be confirmed by combining Δ^9-THCV with other cannabinoid capable to activate the CB_1 receptor, but the benefits reported also for CB_1 receptor agonists (Segovia et al., 2003; Morgese et al., 2007; Martinez et al., 2012) make difficult that such experiment may be explanatory. A second option, not excluding the first one, is that anti-dyskinetic effects of Δ^9-THCV may depend on the activation of CB_2 receptors for which this phytocannabinoid is an agonist, but confirming this option would require additional experiments combining Δ^9-THCV with a selective CB_2 receptor antagonist. Anyway, the option of the CB_2 receptor as a target for the anti-dyskinetic effects of Δ^9-THCV is reinforced by the recent demonstration that HU-308, a synthetic cannabinoid that acts as a selective agonist for the CB_2 receptor, works also against L-DOPA-induced dyskinesia (Rentsch et al., 2020). This option is also supported by the recent data demonstrating that the neuroprotective effects of CB_2 receptor activation in *in vivo* and *in vitro* models of experimental parkinsonism involved the modulation of intracellular signaling related to Pitx3 (He et al., 2020), just the transcription factor used to generate our experimental model. Anyway, it is important to remark that these two options (blocking CB_1 and activating CB_2) are not self-excluding, and may work cooperatively to reduce L-DOPA-induced dyskinesia. This is supported by recent evidence showing that both CB_1 and CB_2 receptors are associated in heteromeric receptor complexes in L-DOPA-induced dyskinesia (Navarro et al., 2018). The interest of this association is that it may affect the pharmacology of both receptors when they work separately. Lastly, a third option for the anti-dyskinetic action of Δ^9-THCV is the possibility that it can target other receptors, *e.g.* GPR55, for which this phytocannabinoid has been also proposed as a potential agonist (Morales et al., 2017), but this would require additional experiments with antagonists for this orphan receptor, which, at present, are not particularly useful.

5. Conclusions

In summary, our data support the anti-dyskinetic potential of Δ^9-THCV to ameliorate adverse effects caused by L-DOPA, in particular delaying the occurrence and attenuating the magnitude of dyskinetic signs. This adds to its promising symptom-alleviating and neuroprotective properties described previously (García et al., 2011). Although further studies are clearly required to determine the clinical significance of these data in humans, the results nevertheless situate Δ^9-THCV in a promising position for developing a cannabinoid-based therapy for PD patients.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2020.104892.

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Authors' contributions

Study design, coordination and supervision: RM, CG and JF-R

Animal treatment, behavioral recording, sampling and histological and qPCR analysis: IE, EK, CP-G and SB

Statistical analysis of the data: IE, RM and JF-R

Manuscript preparation; RM and JF-R with the revision and approval of all authors

Declaration of Competing Interest

Authors declare that they have not any conflict of interest in relation with this submission.

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