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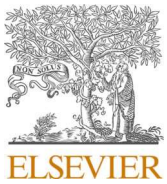

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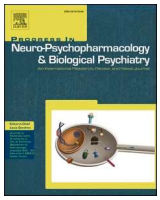

PPARγ receptors are involved in the effects of cannabidiol on orofacial dyskinesia and cognitive dysfunction induced by typical antipsychotic in mice

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ABSTRACT

Tardive dyskinesia (TD) is a movement disorder that appears after chronic use of drugs that block dopaminergic receptors such as antipsychotics. Besides the motor symptoms, patients with TD also present cognitive deficits. Neuroinflammatory mechanisms could be involved in the development of these symptoms. A previous study showed that cannabidiol (CBD), the major non-psychotomimetic compound of *Cannabis sativa* plant, prevents orofacial dyskinesia induced by typical antipsychotics by activating peroxisome proliferator-activated receptors gamma (PPARγ). Here, we investigated if CBD would also reverse haloperidol-induced orofacial dyskinesia and associated cognitive deficits. We also verified if these effects depend on PPARγ receptor activation. Daily treatment with haloperidol (3 mg/kg, 21 days) increased the frequency of vacuous chewing movements (VCM) and decreased the discrimination index in the novel object recognition test in male Swiss mice. CBD (60 mg/kg/daily) administered in the last 7 days of haloperidol treatment attenuated both behavioral effects. Furthermore, haloperidol increased IL-1β and TNF-α levels in the striatum and hippocampus while CBD reverted these effects. The striatal and hippocampal levels of proinflammatory cytokines correlated with VCM frequency and discrimination index, respectively. Pretreatment with the PPARγ antagonist GW9662 (2 mg/kg/daily) blocked the behavioral effects of CBD. In conclusion, these results indicated that CBD could attenuate haloperidol-induced orofacial dyskinesia and improve non-motor symptoms associated with TD by activating PPARγ receptors.

1. Introduction

Tardive dyskinesia (TD) is a hyperkinetic movement disorder that can appear after months or, more commonly, years of the use of drugs that reduce dopaminergic neurotransmission such as reserpine (vesicular depletion) and antipsychotics (D_2 receptors antagonism) (Meyer, 2016). It is characterized by abnormal, involuntary and repetitive movements that affect mainly orofacial region and, in lesser degree, trunk and limbs (Vijayakumar and Jankovic, 2016).

Besides the motor symptoms, cognitive dysfunctions can also be observed in this disorder. Patients with schizophrenia who developed TD present more significant cognitive impairment than those without this motor disorder (Waddington and Youssef, 1996; Wu et al., 2013). In rodents, orofacial dyskinesia induced by haloperidol or reserpine was accompanied by memory deficits (Grover et al., 2013; Naidu et al., 2006; Peres et al., 2016; Thakur et al., 2015).

The pathophysiology of TD is not completely understood yet, but several studies point to the involvement of inflammatory mechanisms. Indeed, increased levels of proinflammatory cytokines were found in the serum and striatum of patients and rats with orofacial dyskinesia, respectively (An et al., 2015; Bishnoi et al., 2008a, 2008b; Peroza et al., 2016). Moreover, inflammatory mediators have been proposed to play a role in the dysregulation of cognitive function (Fourrier et al., 2019). However, it remains unknown if the cognitive dysfunction observed in TD is also related to neuroinflammation.

Cannabidiol (CBD), the major non-psychotomimetic compound of *Cannabis sativa* plant, exhibits anti-inflammatory properties (Burstein, 2015; Campos et al., 2016). In addition to not inducing catalepsy in rodents (Gomes et al., 2013; Moreira and Guimarães, 2005; Sonego et al., 2016; Zuardi et al., 1991) or extrapyramidal effects in humans

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(Leweke et al., 2012), this phytocannabinoid attenuates catalepsy (Gomes et al., 2013; Sonego et al., 2016) and prevents orofacial dyskinesia (Sonego et al., 2018) induced by haloperidol. On the other hand, CBD also reduces cognitive impairments observed in animal models of schizophrenia (Gomes et al., 2015; Murphy et al., 2017; Osborne et al., 2017; Rodrigues da Silva et al., 2020) and Alzheimer's disease (Cheng et al., 2014; Martín-Moreno et al., 2011).

Several pharmacological targets have been associated with CBD effects (Fernández-Ruiz et al., 2012; Izzo et al., 2009). Among them, it can act as an agonist of peroxisome proliferator-activated receptors gamma (PPARγ; O'Sullivan et al., 2009). CBD effects on preventing orofacial dyskinesia (Sonego et al., 2018) and its anti-inflammatory properties (Esposito et al., 2011; Sonego et al., 2018) seem to depend on PPARγ activation. However, it remains to be investigated if these receptors are involved in the cognitive effects of CBD. Corroborating this possibility, the PPARγ agonist pioglitazone reduced the memory deficits and orofacial dyskinesia induced by haloperidol (Grover et al., 2013).

Based on these findings, we hypothesized that CBD could reverse the motor and cognitive symptoms induced by haloperidol *via* PPARγ receptors. To address this question, we investigated if this phytocannabinoid would reduce orofacial dyskinesia and cognitive dysfunction caused by chronic treatment with haloperidol in mice. We also verified if these effects involve neuroinflammatory mechanisms and PPARγ receptor activation.

2. Material and methods

2.1. Animals

The experimental procedures were performed with male Swiss mice (10 weeks old) from Central Animal Facility of the Medical School of Ribeirão Preto, University of São Paulo. The animals were housed in groups of 5 mice/cage, with water and food *ad libitum*, in a temperature-controlled (24 ± 1 °C) room at the Animal Care Unit of Department of Pharmacology and under 12 h light cycle (lights on at 7 am). All procedures were approved by the Local Ethical Committee (CEUA, Medical School of Ribeirão Preto, protocol number: 090/2015), which is under the international laws and policies.

2.2. Drugs

Haloperidol (HAL, typical antipsychotic; Teuto, Brazil) was diluted in saline (vehicle) while cannabidiol (CBD; THC Pharma, Germany) and GW9662 (PPARγ antagonist; Tocris Bioscience, USA) were diluted in 2% Tween 80 in saline (vehicle). All drugs were administered intraperitoneally (ip) in a volume of 10 mL/kg. The drug doses were based on those effective in a previous study using similar protocols (Sonego et al., 2018).

2.3. Experimental design

2.3.1. Experiment 1: Effects of CBD on orofacial dyskinesia and cognitive impairment induced by haloperidol

Animals received a daily injection of haloperidol (3 mg/kg; Sonego et al., 2018) or vehicle during 21 days. In the last 7 days of this period, they received an additional CBD injection (60 mg/kg; Sonego et al., 2018) or vehicle 30 min after haloperidol injection. The frequency of vacuous chewing movements (VCM) was measured on days 0, 14 and 22 of treatment for evaluating orofacial dyskinesia. The novel object recognition (NOR) test was carried out on days 21 and 22 to measure long-term memory. After the behavioral tests, animals were decapitated and prefrontal cortex, striatum and hippocampus were dissected for posterior measurement of cytokines by ELISA (Fig. 1A).

2.3.2. Experiment 2: Acute effect of haloperidol and CBD on long-term memory

In the experiment 1, the last injections of haloperidol and CBD were administered after the acquisition session of the NOR test. Thus, to rule out an acute interference of these drugs on the NOR test, an independent group of animals received after the acquisition session haloperidol (3 mg/kg) or vehicle followed, 30 min later, by CBD (60 mg/kg) or vehicle. The retention session of the NOR test was performed in the next day (Fig. 1B).

2.3.3. Experiment 3: Involvement of PPARγ receptors in the CBD effects on orofacial dyskinesia and cognitive impairment induced by haloperidol

For evaluating the participation of PPARγ receptors in the behavioral

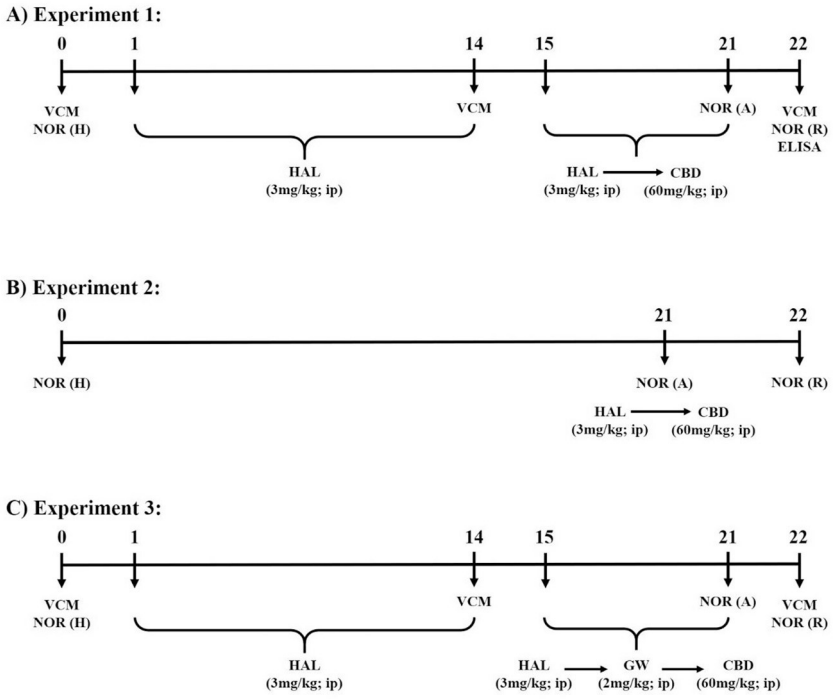

Fig. 1. Experimental design. Animals were treated with haloperidol (HAL) for 21 days. In the last 7 days of this treatment, they received an additional CBD injection 30 min after haloperidol treatment (A). Orofacial dyskinesia (VCM) was evaluated on days 0, 14 and 22 of treatment, while cognitive impairment was tested on days 21 and 22 (NOR). In the experiment with PPARγ antagonist (C), GW9662 was administered 30 min before CBD. To evaluate the acute effect of these drugs on long-term memory, an independent group of animals received, after acquisition session of NOR test, an injection of HAL and, 30 min later, CBD (B). VCM: vacuous chewing movements; NOR: novel object recognition test; (H): habituation; (A): acquisition session; (R): retention session.

effects of CBD, the same protocol of experiment 1 was performed. However, in the last 7 days of haloperidol treatment, animals received GW9662 (2 mg/kg; Sonego et al., 2018) or vehicle 30 min before CBD (Fig. 1C).

2.4. Behavioral tests

2.4.1. Assessment of orofacial dyskinesia

On the test day, mice were placed individually in a glass cylinder (height = 14 cm and diameter = 10 cm) on an acrylic basis with mirrors diagonally arranged outside the cylinder. After 5 min of acclimatization to the apparatus, the frequency of VCM was counted during 10 min. VCM can be defined as a single mouth opening not directed towards any material (Bishnoi et al., 2008b; Grover et al., 2013; Sonego et al., 2018).

2.4.2. Assessment of cognitive dysfunction

Cognitive impairment was evaluated by the NOR test, which was carried out in a Plexiglas circular arena (height = 40 cm and diameter = 40 cm). On day 0 of treatment, immediately after the basal measure of VCM, animals were habituated in the arena for 15 min. On day 21 of treatment, they returned to the arena in the presence of 2 identical objects (acquisition session), and the exploration time of each object was measured. Twenty-four hours later, after assessing orofacial dyskinesia, the retention session was performed for 5 min. In this session, one of the identical objects (familiar) was replaced by a different object (novel) in color, shape, and texture. The time spent by the mouse directing its face to the object in a distance of approximately 2 cm while watching, licking, sniffing, and touching it with the forepaws, was measured. Recognition memory was evaluated through the discrimination index, which was calculated by the difference between the time exploring the novel and the familiar object, corrected for the exploration time of both objects: (novel – familiar)/(novel + familiar; Gomes et al., 2015; Rodrigues da Silva et al., 2020).

2.5. ELISA

Samples were homogenized in PBS 0.1 M and protease inhibitor (Sigma-Aldrich, USA) and were centrifuged at 12000 rpm for 10 min at 4 °C. Their supernatant was collected and kept at −80 °C. The proinflammatory cytokines IL-1β, TNF-α and IL-6 and the anti-inflammatory cytokine IL-10, were quantified using ELISA DuoSet Mouse IL-1 beta, TNF-alpha, IL-6 and IL-10 kits (R&D Systems, USA), respectively, according to manufacturer's instructions. The cytokines levels were corrected by the protein concentration, which was estimated by the Bradford method.

2.6. Statistical analysis

According to the experimental design, data were analyzed by one or two-way of analysis of variance (ANOVA). Post-hoc comparations were performed by Student-Newman-Keuls (SNK) test. Student's *t*-test compared results from acquisition phase of the NOR test. Pearson's correlation was used to evaluate possible associations between the behavioral and the neuroinflammatory results. Statistical results with $p < 0.05$ were considered significant.

3. Results

3.1. Experiment 1: Effects of CBD on orofacial dyskinesia and cognitive impairment induced by haloperidol

Haloperidol increased VCM frequency on day 14 ($F_{6,108} = 6.722$, $p < 0.001$; Fig. 2A), indicating that this typical antipsychotic induced orofacial dyskinesia in the animals. This effect persisted and increased on day 22 (SNK, $p < 0.05$; Fig. 2A). Co-treatment with CBD reduced VCM frequency (SNK, $p < 0.05$; Fig. 2A). By itself, CBD did not induce orofacial dyskinesia (SNK, $p > 0.05$; Fig. 2A).

There was no difference among groups in the exploratory time of the identical objects in the acquisition session regarding to the NOR test. Haloperidol significantly decreased the discrimination index ($F_{3,36} = 4.133$, $p = 0.013$; Fig. 2B), an effect that was attenuated by CBD co-treatment (SNK, $p < 0.05$; Fig. 2B).

Cytokines levels were measured in brain areas related to orofacial dyskinesia and memory. In the prefrontal cortex, animals that received haloperidol and CBD showed reduced levels of IL-1β ($F_{1,36} = 18.6$, $p < 0.001$; Fig. 3A) and TNF-α ($F_{1,36} = 10.52$, $p < 0.005$; Fig. 3B) when compared to those treated with vehicle (SNK, $p < 0.05$) or haloperidol (SNK, $p < 0.05$). In the striatum and hippocampus, haloperidol increased IL-1β ($F_{1,36} = 29.23$, $p < 0.001$ and $F_{1,36} = 15.66$, $p < 0.001$; Fig. 3C and E, respectively) and TNF-α ($F_{1,36} = 66.39$, $p < 0.001$ and $F_{1,36} = 28.43$, $p < 0.001$; Fig. 3D and F, respectively) levels. CBD blocked these effects (SNK, $p < 0.05$). No interaction was found between haloperidol and CBD treatment with IL-6 and IL-10 in the striatum and hippocampus (Supplementary Fig. S1).

Moreover, striatal levels of IL-1β ($r = 0.6041$, $p < 0.001$) and TNF-α ($r = 0.6438$, $p < 0.001$) correlated positively with the frequency of VCM. The discrimination index correlated negatively with levels of these cytokines in the hippocampus (IL-1β: $r = -0.4711$; TNF-α: $r = -0.4497$; $p < 0.005$), but not in the prefrontal cortex.

3.2. Experiment 2: Acute effect of haloperidol and CBD on long-term memory

Again, there was no difference among groups in the identical objects' exploratory time in the acquisition session. Haloperidol, CBD, and their combination increased the discrimination index ($F_{3,36} = 6.256$, $p < 0.002$; Fig. 4).

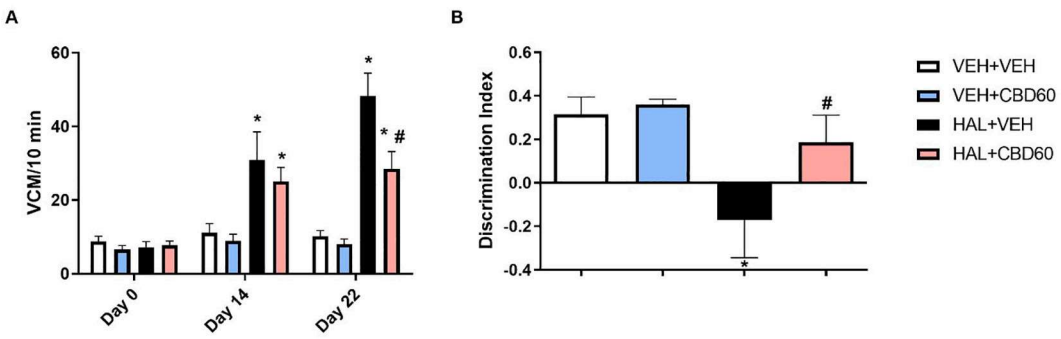

Fig. 2. Effects of the treatment with CBD (60 mg/kg), for 7 days, on orofacial dyskinesia and cognitive impairment induced by haloperidol (HAL, 3 mg/kg) in mice (n = 9–11 animals/group). CBD attenuated the frequency of VCM (A) and long-term memory deficit (B) promoted by this typical antipsychotic. Data presented as mean ± SEM. *$p < 0.05$ from VEH + VEH group, $^{\#}p < 0.05$ from HAL + VEH group.

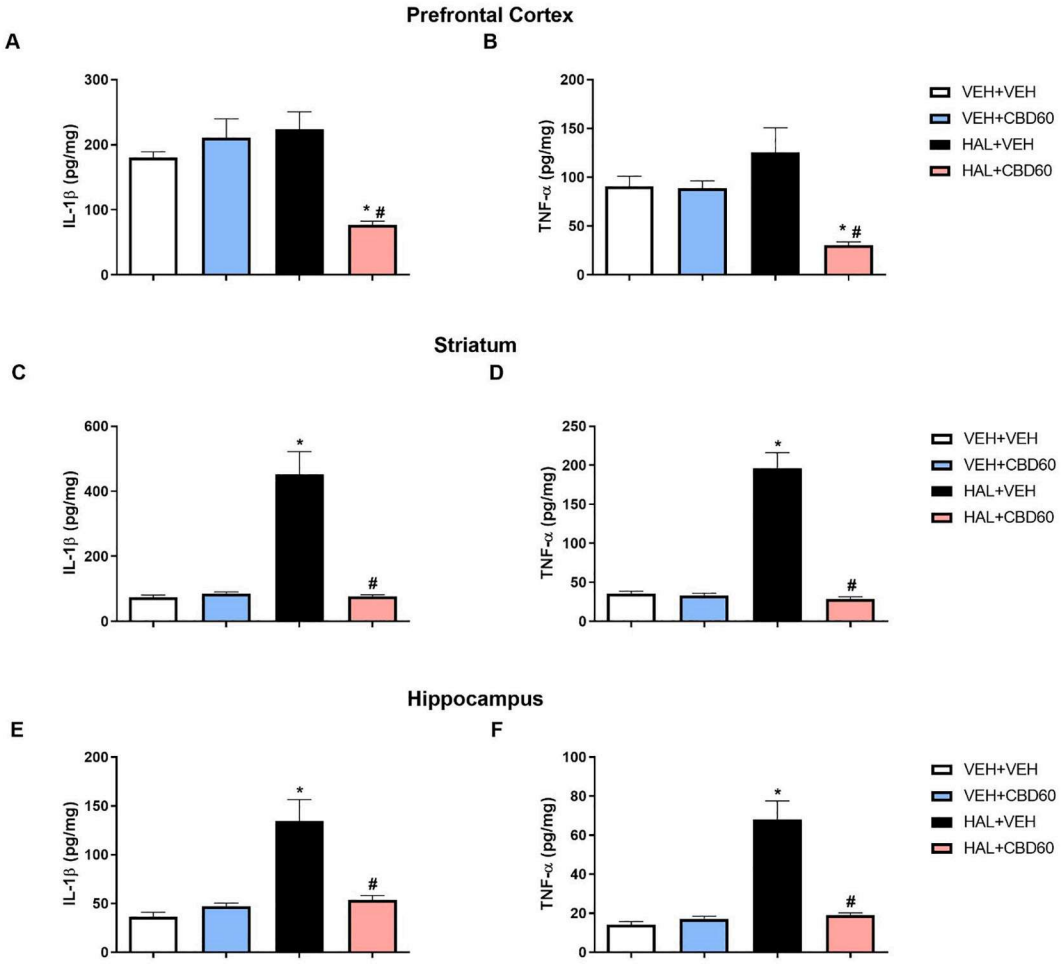

Fig. 3. Effects of the treatment with CBD (60 mg/kg), for 7 days, on proinflammatory cytokine levels induced by haloperidol (HAL, 3 mg/kg) in mice (n = 9–11 animals/group). The combination of haloperidol and CBD reduced the levels of IL-1β (A) and TNF-α (B) in the prefrontal cortex. CBD also reversed the haloperidol-induced cytokines in the striatum (C and D) and hippocampus (E and F). Data presented as mean ± SEM. $^{*}p < 0.05$ from VEH + VEH group, $^{\#}p < 0.05$ from HAL + VEH group.

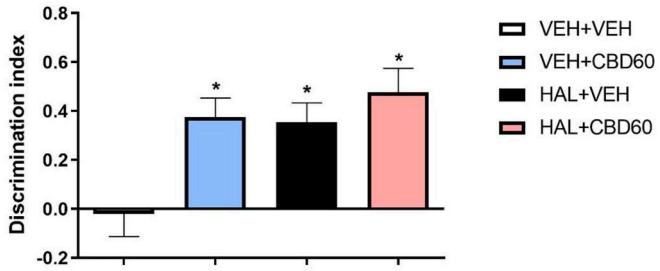

Fig. 4. Effect of the acute treatment with haloperidol (HAL, 3 mg/kg) and CBD (60 mg/kg) on novel object recognition (NOR) test performed in mice (n = 10 animals/group). Haloperidol, CBD and the combination of these drugs improved the long-term memory. Data presented as mean ± SEM. $^{*}p < 0.05$ from VEH + VEH group.

3.3. Experiment 3: Involvement of PPARγ receptors in the CBD effects on orofacial dyskinesia and cognitive impairment induced by haloperidol

Haloperidol increased VCM frequency on day 14 and 22 ($F_{8,96} = 8.172$, $p < 0.001$; SNK, $p < 0.05$; Fig. 5A). CBD reduced VCM frequency on day 22 (SNK, $p < 0.05$; Fig. 5A). The PPARγ antagonist GW9662 blocked the effect of CBD (SNK, $p < 0.05$; Fig. 5A) on orofacial dyskinesia.

Regarding to the NOR test, there was no difference between the exploratory time of the identical objects in the acquisition session. Haloperidol decreased the discrimination index between the familiar and novel object, an effect that CBD attenuated. GW9662 pretreatment prevented CBD effects ($F_{4,32} = 4.012$; $p < 0.05$; SNK, $p < 0.05$; Fig. 5B).

4. Discussion

The present study showed that CBD can block the progression of orofacial dyskinesia induced by haloperidol once it was established. Preclinical and clinical studies have demonstrated the therapeutic potential of CBD for motor disorders (Gomes et al., 2013; Leweke et al., 2012; Sonego et al., 2016; Zuardi et al., 2009). Regarding to TD, this phytocannabinoid prevented the development of orofacial dyskinesia promoted by chronic treatment with haloperidol in mice (Sonego et al., 2018). Moreover, it reduced the frequency of VCM induced by reserpine in rats (Peres et al., 2016). In the present study, we showed that, besides preventing its development, CBD could also decrease VCM frequency once they have appeared along with chronic haloperidol administration.

Repeated, but not acute, haloperidol administration caused a long-term memory deficit, measured by the NOR test. There was, however, a significant difference in the control performance between the acute and repeated treatment experiments. However, in the former, animals were handled and received two injections of the vehicle in a single day. Since stress exposure can cause memory impairment in the NOR (Towers

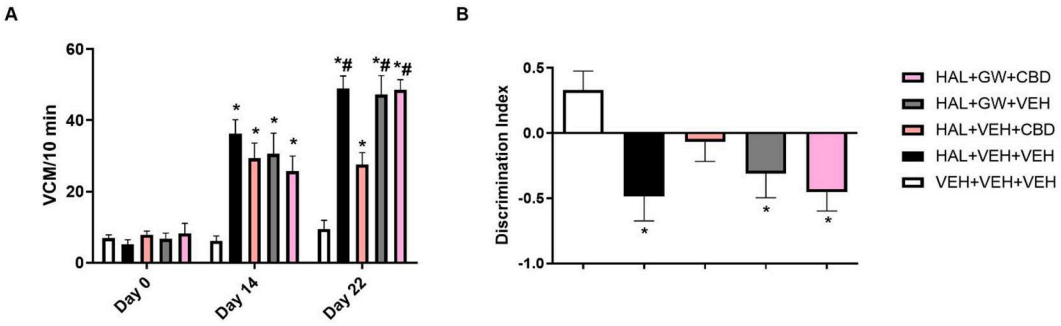

Fig. 5. Involvement of PPARγ receptors (GW9662, 2 mg/kg) in the effects of CBD (60 mg/kg) on orofacial dyskinesia and cognitive impairment induced by haloperidol (HAL, 3 mg/kg) in mice (n = 7–8 animals/group). The PPARγ antagonist blocked the CBD effects on frequency of VCM (A) and discrimination index (B). Data presented as mean ± SEM. $^{*}p < 0.05$ from VEH + VEH + VEH group, $^{\#}p < 0.05$ from HAL + VEH + CBD group.

et al., 2019), tolerance to the handling and injection procedures could be responsible for this difference.

The NOR results agree with studies showing that this typical antipsychotic can induce cognitive impairment (Grover et al., 2013; Thakur et al., 2015). Furthermore, in a traumatic brain injury (TBI) model, rats treated chronically with haloperidol show decreased spatial learning recovery in the Morris water maze. This cognitive impairment was still present 1 and 3 months after the treatment (Phelps et al., 2015).

Like the effects observed in VCM frequency, CBD attenuated the memory deficit observed in this model of TD. This result corroborates those of Peres et al. (2016), showing that this phytocanabinoid prevented cognitive dysfunction induced by reserpine, another model of TD. Pro-cognitive effects of CBD have also been observed in other animal models, such as brain ischemia (Mori et al., 2017) and infection (Barichello et al., 2012; Campos et al., 2015). In common, these models involve neuroinflammatory changes.

In our study, CBD reversed the haloperidol-induced increase of IL-1β and TNF-α levels in the striatum and hippocampus, brain areas related to motor control and memory, respectively (Do et al., 2013; Fourrier et al., 2019). Furthermore, there were significant correlations between the levels of these proinflammatory cytokines and the behaviors evaluated. No effect was observed in the prefrontal cortex. A failure of repeated haloperidol treatment (7 days) to increase cytokine levels in rats has already been reported (Nishigaki et al., 2019). Cytokine levels in controls were much higher in this region than in the striatum. Therefore, a ceiling effect could have decreased the sensitivity to detect a further increase. However, these regional differences could also involve local cellular effects of D2 receptors. Although classically linked to activation of Gi/o and adenylate cyclase inhibition, these receptors have been recently reported to enhance, rather than decrease, the excitability of pyramidal neurons in the prefrontal cortex through a stimulatory G-protein pathway (Robinson and Sohal, 2017).

Haloperidol-induced orofacial dyskinesia has been associated with an increase of IL-1β and TNF-α (Bishnoi et al., 2008a; Peroza et al., 2016). Since these cytokines can induce neurotoxicity (Chao et al., 1995), they could mediate the development of the motor symptoms of TD. Corroborating this possibility, our group demonstrated that the preventive effect of CBD on haloperidol-induced orofacial dyskinesia is associated with reduced production of proinflammatory cytokines and microglial activation in the striatum (Sonego et al., 2018).

IL-β and TNF-α are required for learning and memory processes (Baune et al., 2008; Goshen et al., 2007). However, at supraphysiological levels, these cytokines could promote cognitive dysfunction (Fiore et al., 2000; Goshen et al., 2007). IL-1β and TNF-α, for example, can inhibit the hippocampal long-term potentiation (LTP; Cunningham et al., 1996). This synaptic plasticity has been related to learning and memory (Fourrier et al., 2019). CBD also attenuates hippocampal neurodegeneration and decreases cytokine levels and microglial activation induced by several stimuli (Barichello et al., 2012; Campos et al., 2015; Mori et al., 2017).

The anti-inflammatory effects of CBD could be mediate by PPARγ receptors (Esposito et al., 2011). These receptors belong to the nuclear receptors superfamily (Daynes and Jones, 2002) and regulate metabolic pathways mainly related to adipogenesis and glucose metabolism. In addition, they also produce anti-inflammatory effects. Once activated, PPARγ receptors translocate to the nucleus, where they could associate with transcription factors such as NFκB, STAT and AP-1, inhibiting their transcriptional activity (Daynes and Jones, 2002; Ricote et al., 1998). In the central nervous system (CNS), PPARγ receptors are extensible located in the striatum and hippocampus (Moreno et al., 2004), the two brain areas relevant to this study.

Similar to the present findings, in a previous work, the preventive CBD effect on haloperidol-induced TD was blocked by the PPARγ antagonist GW9662 (Sonego et al., 2018). In the same study, CBD decreased NFκB translocation to the nucleus in microglial cells stimulated by LPS, an effect mediated by PPARγ receptors (Sonego et al., 2018). Pioglitazone, an agonist of these receptors, also reduced the frequency of VCM induced by haloperidol in rats. This effect was accompanied by a decrease of IL-1β and TNF-α levels in the striatum (Grover et al., 2013). Additionally, pioglitazone prevented the memory deficits induced by haloperidol and ameliorated the cognitive impairments observed in APP/PS1 transgenic mice, a model of Alzheimer's disease (Mandrekar-Colucci et al., 2012), and Poly(I:C)-treated offspring, a model of schizophrenia (Zhao et al., 2019). In both models, the cognitive effects of pioglitazone were associated with a reduction of proinflammatory pathway and a increase of anti-inflammatory response in microglial cells (Mandrekar-Colucci et al., 2012; Zhao et al., 2019). Another PPARγ agonist, rosiglitazone, improved short and long-term memory of leptin receptor deficient mice (*db/db*), a model of type 2 diabetes. Moreover, rosiglitazone prevented hippocampal LTP reduced in diabetic mice (Kariharan et al., 2015). In turn, CBD also reversed the reduction of LTP induced by β-amyloid in the hippocampus, an effect blocked by PPARγ antagonist GW9662 (Hughes and Herron, 2019). Taken together, these results suggest that the PPARγ-NFκB pathway could be involved in the anti-inflammatory and anti-dyskinetic effects of this phytocannabinoid.

Long-term use of CBD is usually safe and well-tolerated by most patients (Dos Santos et al., 2020), including older adults (Velayudhan et al., 2021) and those with Parkinson's disease (Leehey et al., 2020; Zuardi et al., 2009). Also, no cognitive impairment has been seen after chronic administration in children and adults with epilepsy (Martin et al., 2019; Thompson et al., 2020).

In conclusion, this study demonstrated that CBD could reduce orofacial dyskinesia induced by chronic treatment of haloperidol once it was established. Moreover, this phytocannabinoid also improves cognitive dysfunction associated with the development of orofacial dyskinesia. CBD effects could be mediated by activation of PPARγ receptors, leading to reduced neuroinflammatory changes in critical areas

related to TD symptoms. Therefore, it could be a new therapeutic approach to prevent the progression of TD symptoms.

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Ethical statement

Procedures were approved by the Local Ethical Committee (CEUA, Medical School of Ribeirão Preto, protocol number: 090/2015), which is under the international laws and policies.

Declaration of Competing Interest

FSG is a co-inventor (Mechoulam R, JC, Guimaraes FS, AZ, JH, Breuer A) of the patent "Fluorinated CBD compounds, compositions and uses thereof. Pub. No.: WO/2014/108899. International Application No.: PCT/IL2014/050023" Def. US no. Reg. 62,193,296; 29/07/2015; INPI on 19/08/2015 (BR1120150164927). The University of São Paulo has licensed the patent to Phytecs Pharm (USP Resolution No. 15.1.130002.1.1). The University of São Paulo has an agreement with Prati-Donaduzzi (Toledo, Brazil) to "develop a pharmaceutical product containing synthetic cannabidiol and prove its safety and therapeutic efficacy in the treatment of epilepsy, schizophrenia, Parkinson's disease, and anxiety disorders".

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2021.110367.

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