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PII: S0028-3908(19)30370-3
DOI: https://doi.org/10.1016/j.neuropharm.2019.107808
Reference: NP 107808

To appear in: Neuropharmacology

Received Date: 22 May 2019
Revised Date: 11 September 2019
Accepted Date: 2 October 2019


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Cannabidiol increases the nociceptive threshold in a preclinical model of Parkinson’s disease

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Abstract

Medications that improve pain threshold can be useful in the pharmacotherapy of Parkinson’s disease (PD). Pain is a prevalent PD’s non-motor symptom with a higher prevalence of analgesic drugs prescription for patients. However, specific therapy for PD-related pain are not available. Since the endocannabinoid system is expressed extensively in different levels of pain pathway, drugs designed to target this system have promising therapeutic potential in the modulation of pain. Thus, we examined the effects of the 6-hydroxydopamine-induced PD on nociceptive responses of mice and the influence of cannabidiol (CBD) on 6-hydroxydopamine-induced nociception. Further, we investigated the pathway involved in the analgesic effect of the CBD through the co-administration with a fatty acid amide hydrolase (FAAH) inhibitor, increasing the endogenous anandamide levels, and possible targets from anandamide, i.e., the cannabinoid receptors subtype 1 and 2 (CB1 and CB2) and the transient receptor potential vanilloid type 1 (TRPV1). We report that 6-hydroxydopamine-induced parkinsonism decreases the thermal and mechanical nociceptive threshold, whereas CBD (acute and chronic treatment) reduces this hyperalgesia and allodynia evoked by 6-hydroxydopamine. Moreover, ineffective doses of either FAAH inhibitor or TRPV1 receptor antagonist potentialized the CBD-evoked antinociception while an inverse agonist of the CB1 and CB2 receptor prevented the antinociceptive effect of the CBD. Altogether, these results indicate that CBD can be a useful drug to prevent the parkinsonism-induced nociceptive threshold reduction. They also suggest that CB1 and TRPV1 receptors are important for CBD-induced analgesia and that CBD could produce these analgesic effects increasing endogenous anandamide levels.

Keywords: 6-hydroxydopamine; anandamide; cannabidiol; cannabinoid receptors; Parkinson’s disease; Pain; Transient receptor potential vanilloid type 1.
Introduction

Besides originally described as a motor disease, Parkinson’s disease (PD) patients suffer from a variety of non-motor symptoms such as sleep disorders, olfactory dysfunctions, anxiety, depression, and pain (Dauer and Przedborski, 2003; Faivre et al., 2019). All these symptoms have a significant impact on their quality of life (Aarsland and Kramberger, 2015; Calabresi et al., 2006; Nègre-Pagès et al., 2008), and usually appear a long time before the first motor signals (Bezard and Fernagut, 2014; Blanchet and Brefel-Courbon, 2018).

Around 60% of PD patients are affected by pain (Barone et al., 2009; Faivre et al., 2019; Politis et al., 2010), which can be directly or partly related to the disease. It can appear as nociceptive, neuropathic, or miscellaneous pain (Wasner and Deuschl, 2012). Also, compared to healthy controls, these patients have lower pain threshold and tolerance (Blanchet and Brefel-Courbon, 2018), developing allodynia (Djaldetti et al., 2004; Schestatsky et al., 2007). However, the mechanisms of PD-associated pain are not completely understood, and this problem has not received much attention from preclinical researchers.

Decreased nociceptive threshold has been reported in rodent models of PD after 1-methyl-4-phenyl-1,2,4,5-tetrahydropyridine (MPTP) or 6-hydroxydopamine (6-OHDA)-induced dopaminergic degeneration (Gómez-Paz et al., 2018; Nascimento et al., 2018; Zengin-Toktas et al., 2013). These findings suggest that the loss of dopamine (DA) in the basal ganglia may be involved in the reduced pain threshold (Wasner and Deuschl, 2012).

The degeneration of the nigrostriatal pathway impairs the dopamine-mediated descending pathways, resulting in hyperalgesia (Fil et al., 2013). In this way, Magnusson and Fisher (2000) showed that systemic administration of DA agonists induced hypoalgesia by modulating D2 receptors in the dorsolateral striatum (Magnusson and Fisher, 2000). Furthermore, some studies have pointed out to an increase in the pain threshold after administration of the dopaminergic medication such as levodopa (Brefel-Courbon et al., 2005; Gerdelat-Mas et al., 2007). However, the absence of a total recuperation of these symptoms with dopaminergic therapy suggests that non-dopaminergic mechanisms could also be involved in the appearance or maintenance of pain symptoms (Brefel-Courbon et al., 2005).

Despite the higher prevalence of analgesic drugs prescription for PD patients (33%) than for general population (20%), pain is often neglected and insufficiently treated in these patients (Broetz et al., 2007; Blanchet and Brefel-Courbon, 2018). In addition, analgesic drugs frequently cause side effects such as opiate-induced constipation that in turn may exacerbate constipation developed by parkinsonian patients in early stages of the disease (Stocchi and
Torti, 2017). Therefore, even though the well-established correlation between pain and PD, still there is no specific therapy for the Parkinson's disease-related pain, thus requiring further efforts in investigating effective drugs, with fewer side effects, for pain in this neurodegenerative condition (Seppi et al., 2019).

The endocannabinoid system could be a promising target for the treatment of PD-associated pain. This system has now been recognized as presenting great therapeutic potential in the modulation of pain (Guindon and Hohmann, 2009), besides having a neuroprotective effect on neurodegenerative diseases (Chagas et al., 2014). Cannabidiol (CBD) is the principal non-intoxicating phytocannabinoid constituent of the Cannabis sativa plant (Shohami et al., 2011; Russo, 2017). CBD has low affinity for both cannabinoid subtype 1 (CB1) and 2 (CB2) receptors (Ligresti et al., 2016), but it can upregulate the levels of the endocannabinoid anandamide (AEA) by inactivation of the Fatty Acid Amide Hydrolase (FAAH) (Campos et al., 2010), the enzyme responsible for anandamide degradation.

Besides AEA effects in cannabinoid receptors, AEA is also an agonist of the transient receptor potential vanilloid type 1 (TRPV1) (Campos et al., 2010; dos-Santos-Pereira et al., 2016; Zygmunt et al., 2000). TRPV1 is a channel activated by multiple painful stimuli including noxious heat, pungent chemicals (capsaicin and jellyfish venom), and protons (Kaneko and Szallasi, 2014; Szallasi et al., 2007). Accumulated compelling data have demonstrated the role of TRPV1 in inflammatory and neuropathic pain states (Moran and Szallasi, 2018; Stocchi and Torti, 2017). For instance, TRPV1 activation in nociceptive neurons triggers the release of neuropeptides and transmitters, resulting in perceived pain (Jara-Osegueda et al., 2010).

Therefore, CBD may represent a useful pharmacological alternative in the treatment of PD-related pain. Here, we tested the hypothesis that CBD has an antinociceptive effect in a preclinical model of 6-OHDA-induced parkinsonism in mice via FAAH inhibition and indirect activation of the CB1 receptor.

2. Materials and Methods

2.1. Ethical statement: Male adult C57BL6 mice (FMRP-USP, Ribeirão Preto, Brazil; 20–25g body weight) were used in this study. All animal experimental procedures were approved by the local Animal Care and Use Committee of the University of São Paulo/Brazil at the Ribeirao Preto campus (Protocol number # 2017.1.369.58.4) and are in accordance with the Guide for the Care and Use of Laboratory Animals of the National Council for the Control
of Animal Experimentation (CONCEA).

2.2. Experimental animals - Housing and husbandry: C57/BL6 mice were housed in groups of three animals per cage in a controlled environment under a light-dark cycle of 12 hours (lights on at 06:00 and off at 18:00h), in a quiet room with controlled temperature (23 °C ± 1 °C). All animals had free access to food and water. Animal care, including the manual recording of body weight, observation of food and water intake, and cage changes that occurred daily during the middle of the light cycle. Mice were left undisturbed during their dark cycle.

2.3. Pharmacological treatments: The following drugs, at doses based on the literature, were used: cannabidiol (CBD, 10, 30 and 100 mg/kg (McPartland et al., 2015)), morphine (MOR, 10 mg/kg (Aksu et al., 2018)), celecoxib (CXB, non-steroidal anti-inflammatory, 20 mg/kg, (Zhao et al., 2017)), URB597 (URB, FAAH inhibitor, 0.5 mg/kg, (Campos et al., 2013)), AM251 (AM, CB1 antagonist, 1 mg/kg, (Campos et al., 2013)), Capsazepine (CPZ, TRPV1 antagonist, 5 mg/kg, (dos-Santos-Pereira et al., 2016)) and SCH 336 (SCH, CB2 inverse agonist, 2mg/kg, (Lunn et al, 2006). All drugs were administered intraperitoneally.

2.4. Sample size: The total number of mice used in this work was 49. All mice were tested in the nociception tests before and after 6-OHDA lesion for determination of hyperalgesia and allodynia time-course (first experimental protocol). Then, they were randomly distributed to vehicle (VEH, n=6), CXB (n=6), MOR (n=6), CBD (n=7 per each dose) groups for the second experimental protocol. After a 3-day drug washout, these mice were randomly redistributed to the following groups: VEH (n=9), URB (n=10), AM (n=10), CPZ (n=10) and SCH (n=10) treatments. Finally, one-week drug washout was performed, and 32 mice were randomly distributed to VEH (n=7), CBD (n=8), CBD+URB (n=8), CBD+AM (n=8), CBD+CPZ (n=8) or CBD+SCH (n=8) groups. In all cases, the animals were re-tested in the nociception tests to ensure drug washout.

2.5. Drug treatment and experimental design: This work was compounded by four experimental protocols:

   (1) First, nociceptive tests were performed before the 6-OHDA lesion (-1 day) and after 7, 14, and 21 days of the surgery.
After 21 days of the surgery, these mice were distributed into VEH, CXB, MOR and CBD10, CBD30 and CBD100 mg/kg for the second experimental protocol. This protocol consisted in evaluating CBD effects on hyperalgesia (by Hot Plate and Tail Flick tests) and allodynia (Acetone Drop and Von Frey tests) responses compared to effects of celecoxib, morphine and saline administration in mice with established motor impairment (Data not shown).

After 3-day of drug washout, we tested the antinociceptive effects of chronic treatment of CBD. Mice received CBD (10 mg/kg) for 14 days, once a day, and then were tested for nociceptive behaviors 60 minutes after the last administration.

After 3-day of drug washout, we tested different doses of URB, AM, CPZ and SCH to find ineffective doses of these drugs on hyperalgesia and allodynia responses in parkinsonian mice. These ineffective doses were used in the fourth protocol.

Finally, after one week of drug washout, we test the involvement of FAAH, CB1, CB2 and TRPV1 in the antinociceptive effect of the CBD in parkinsonian mice. A pre-treatment with URB, AM, CPZ or SCH was made 30 min before CBD administration (30 mg/kg – the least effective dose according to our results). Hyperalgesia and allodynia tests were performed 30 min after CBD administration.

After each protocol and drug washout, animals were re-subjected to nociception tests to ensure drug washout (data not shown) and afterward regrouped randomly for performing the following protocol.

2.6. Experimental Procedures: All tests were performed at the light phase, in the morning period (8:00-12:00) and at the same place (in the same laboratory). The same trained person performed all the nociception tests. The information about the treatments was withheld from experimenters that performed the hyperalgesia and allodynia tests to reduce bias, thus characterizing a blinded-experiment.

26.1. 6-Hydroxydopamine-lesioned mice model: The 6-OHDA-induced lesion was based on a previous study (dos-Santos-Pereira et al., 2016). 6-Hydroxydopamine (6-OHDA; 2,4,5-Trihydroxyphenethylamine hydrochloride; 2.5 ug/ul diluted in saline 0.9% containing 0.02% ascorbic acid) obtained from Sigma-Aldrich, USA, was used. Third-two animals were anesthetized with 2,2,2-tribromoethanol (250 mg/kg i.p.) and fixed in the stereotaxic apparatus for performing the surgery (David Kopf, model USA, 9:57). Stereotaxic coordinates of the dorsolateral region of striatum were (in mm from Bregma): anteroposterior
(AP) = +0.5; lateral-lateral (LL) = -2.3 and dorsoventral (DV) = −3.9 and −3.0 (Paxinos and Franklin, 2001). 6-OHDA-HCl was microinjected in a total volume of 2 µl/injection (2 injection places; 1 µL/min). After the microinjection, the cannula was left in place for five additional minutes to prevent reflux of the injected solution. At the end of the surgical procedure, the animals were kept warm by a 60W light bulb until full recovery from anesthesia and then led to the vivarium, where they received amoxicillin (5 mg/ml orally) for three days.

2.6.2. Cylinder test: Animals were placed in a clear plastic cylinder (3.8 inches wide, 6 inches tall) for 2 min, and the number of times they touch the cylinder wall with the right, left, or both forepaws following rearing was recorded (Schallert et al., 2000). The asymmetry score was calculated as the sum of left (unaffected) forepaw touches + 1/2 bilateral touches/total number of forepaw touches (right + left + bilateral). For the mice that use both forepaws equally, the score would be 0.5. For mice with the complete asymmetry of forepaw use due to severe dopamine depletion affecting the right forepaw, the asymmetry score would be 1 (Data not shown).

2.6.3. Nociceptive Behaviors: The nociceptive tests were performed according to the following sequence: Von Frey, Acetone Drop, Tail Flick, and Hot Plate. This sequence was based on previous studies from our group (do Nascimento and Leite-Panissi, 2014; Nascimento et al., 2018). Specifically, the hot plate test was designed to evaluate the supraspinally-integrated responses (Caggiula et al., 1995; Rubinstein et al., 2002), and it determines changes in latency as an indicator of modifications of the supraspinal pain process. In turn, the tail-flick latency is mediated by a spino-bulbo-spinal circuit (Le Bars et al., 2001), as well as acetone – cold test, which is compared with the tail-flick test, but it uses the paw withdrawal as parameter, avoiding the use of the tail that is an important organ related to thermoregulation. In addition, different effects could be found with mechanical von Frey filament, an important instrument to access the mechanisms of cutaneous stimulation-induced sensory input (Pitcher et al., 1999).

2.6.3.1. Hot Plate: Thermal hyperalgesia was assessed with the hot plate test as previously described (Fujii et al., 2019). Mice were placed in a 10-cm wide glass cylinder in a hot plate apparatus (Insight, Wembley, UK) at 55±0.3 °C. The end-point (latency) was characterized by the removal of the paw, followed by clear paw flinching or licking movements. A cut-off of 30 s was established to prevent tissue damage (Kinsey et al., 2011). The latency of the flick
response was measured in seconds, and each animal was tested three times with 60 seconds between each trial. The scores were averaged over the 3 trials for the final score of each animal.

2.6.3.2. Tail Flick: The spinally mediated thermal hyperalgesia was assessed by the tail-flick test as described previously (Nascimento et al., 2018). Mice were placed at the apparatus where a thermal stimulus was applied to the tail. We measured the latency for tail withdrawal as an indication of the pain threshold. A cutoff time of 9-s was used to prevent tissue damage. The latency of flick response was measured in seconds, and each animal was tested three times, with 60 seconds between each trial. These scores were averaged over the 3 trials for the final animal score. To avoid novelty stress-induced analgesia, animals were acclimated to the test situation two days before the experiment by restraining the mouse in the same apparatus, but without the thermal stimulus.

2.6.3.3. Acetone Drop Test: Cold allodynia was assessed by the modified acetone drop test as described previously (Brenner et al., 2014). Mice were acclimated for 5 min on a wire mesh. Then, we sprayed acetone (100 µL) onto the plantar surface of the hind paw, without touching the skin. The first 10 seconds of activity were disregarded as a response to the direct application of the droplet. We measured the flinches in response to skin cooling induced by acetone using the following scores: 0- none; 1- rapid withdrawal or abrupt movement of the paw; 2- prolonged withdrawal or repeated movements of the paw; 3- repeated movements followed by licking of the paw.

2.6.3.4. Von Frey test: Mechanosensitivity can be determined as the threshold amount of force required to elicit a behavioral response, such as the withdrawal of a paw from the applied stimulus (Chaplan et al., 1994; Nascimento et al., 2018, 2013). Employing von Frey filaments (Nylon monofilaments - 0.35 mm diameter, Scientific Anglers™ Full Sinking Fly Fishing Line) from 0.01 to 15.1 g, we first characterized the percent response at each stimulus intensity (calibration). In the experimental day, the mice were placed individually in a cage (10 X 20 X 15 cm) with a meshed metal wire floor (squares of 5 X 5 mm). Mice were successively stimulated starting with the weakest to the strongest filaments (0.05 to 4 g). The sequence of stimuli stopped when the animal reacted with immediate flinching or licking of the hind paw. The force of the last used filament was considered the pain threshold.
2.7. **Euthanasia and Tissue Processing:** At the end of the behavioral analysis, the animals were deeply anesthetized with urethane (1.5 g/kg, Sigma-Aldrich, St. Louis, MO, USA) and rapidly perfused transcardially with cold 0.9% saline solution containing heparin (200 µl of heparin 25,000 UI/l of solution) and sodium nitrite (1 g/l solution). Animals were then immediately perfused with 4% paraformaldehyde (PFA; pH 7.4; Sigma-Aldrich, St. Louis, MO, USA). Brains were immediately removed, post-fixed in 4% paraformaldehyde for 2 h, and cryoprotected in 30% sucrose solution. Brains were quickly frozen in isopentane (−40 °C, Sigma-Aldrich, St. Louis, MO, USA) and stored at −80 °C until histological processing. Coronal sections (25 µm) were processed in a freezing microtome (Leica, model CM1850) throughout the rostrocaudal extent of the striatum and substantia nigra compacta. Striatal sections were obtained from rostral (Bregma: +0.86 mm), medial (Bregma +0.02 mm) and caudal areas (Bregma −0.58 mm). Substantia nigra sections were obtained from rostral (Bregma −2.54 mm), medial (Bregma −3.16 mm) and caudal areas (Bregma −3.80 mm).

2.8. **Immunohistochemistry:** To verify the extension of 6-OHDA lesion, immunostaining was then carried out in free-floating sections with standard avidin–biotin immunohistochemical protocols (Gomes et al., 2008) with specific tyrosine hydroxylase (TH, 1:2000; Pel Freez, Arkansas, USA) antibody diluted in 0.1 M phosphate-buffered saline (pH 7.4), containing 0.15% Triton X-100. After incubation with the primary (48 h) and secondary (1.5 h) antisera, peroxidase reactions were developed with diaminobenzidine. The slices were mounted on slides and coverslipped for microscope observations. Samples from each experimental group were processed at the same time. Quantitative analyses were then performed in striatum and substantia nigra.

2.9. **Statistical Analysis:** The effect on the time course of 6-OHDA-induced nociception was evaluated by repeated measures analysis of variance (r-ANOVA), with drug and time as factors. The results are expressed as mean ± S.E.M, and post-hoc analysis was performed with the help of the Bonferroni’s test. For the analysis of the nociception tests and immunohistochemistry, comparison between different groups was performed using two-way analysis of variance (ANOVA) followed by the Tukey’s multiple comparisons test. Values are presented as mean ± S.E.M. Statistical significance was set at p<0.05. All statistics in this study were performed using Prism 6, version 6.0d (GraphPad Software, Inc.).
3. Results

3.1. Time-course of 6-OHDA-induced hyperalgesia and allodynia responses

In the present study, we first confirmed by immunohistochemistry that 6-OHDA induced PD-like lesions (Figure 1A). We used TH-immunoreactivity (TH-ir) to assess the percentage of the striatal area devoid of TH-ir for all mice used in the experiments. On average, 65% of the striatum was denervated. There was a lesion side effect for all striatal analyzed areas (rostral: $F_{2,42} = 3.3; p<0.05$; medial: $F_{2,38} = 12.7; p<0.05$; caudal: $F_{2,48} = 6.4; p<0.05$, data not shown).

To rule out that different lesion intensity would interfere with CBD analgesic effects, the cylinder test was performed. The ratio of forelimb placement [(left - right)/(right + left + both)] of the non-treated group did not differ from drug treatment group ($F_{4,42} = 4.2; p>0.05$).

As expected, there was no interaction “lesion side × drug treatment” ($F_{4,48} = 13.1; p>0.05$, Supplementary Data).

We used four nociceptive behavioral tests to measure the time-course of thermal and mechanical pain threshold in mice after 6-OHDA-injection. The hot plate test showed a significantly decrease in thermal latency in mice at 14 and 21 days from surgery ($F_{4,48} = 3.1; p<0.01$; Figure 1C) when compared to basal measures. On the other hand, tail flick latency diminished since the first analyzed period (7 – 21 days) after 6-OHDA lesion ($F_{4,48} = 6.4; p<0.05$; Figure 1D). Regarding allodynia responses, there was significantly increase in nociception behavior at 14 and 21 days from surgery when compared to basal measures in both tests (acetone drop, $F_{4,48} = 13.3; p<0.05$; Figure E; and von Frey, $F_{4,48} = 4.3; p<0.05$; Figure F).

These results suggest that the mice model of PD induced by unilateral lesion of the striatum displays thermal and mechanical pain hypersensitivity that start at different time points after the 6-OHDA lesion.

3.2. Antinociceptive effects of acute CBD on experimental parkinsonism

Hyperalgesia response – Twenty-one days after 6-OHDA injection, we tested the antinociceptive effects of CBD. Three different doses (10, 30, 100 mg/kg i.p.) of CBD were administered 60 min previously to the nociceptive tests. CXB, MOR, and CBD (10, 30 and 100 mg/kg) increased hot plate response latency ($p<0.05$; Figure 2A) when compared to VEH group. Morphine (MOR) also significantly increased this latency when compared to CXB and the three doses of CBD ($F_{3,48} = 5.31 p<0.01$; Figure 2A). In addition, the highest dose of CBD...
(100 mg/kg) induced antinociceptive effects in the hot plate test comparing to the other two
doses (F_{3,32} = 6.8; p<0.05; Figure 2A). The tail flick latency was measured to evaluate a
hyperalgesia response under spinal control. The results indicated that celecoxib (CXB),
morphine (MOR) and the three doses of CBD significantly reduced this thermal hyperalgesia
when compared to VEH group (F_{3,48} = 17.3; p<0.05; Figure 2B). The highest dose of CBD
(100 mg/kg) was the most effective in increasing tail flick latency.

**Allodynia response** – There was no change in the withdrawal latency to cold stimulus
in the VEH, CXB, and CBD (30 mg/kg) mice groups (p>0.05; Figure 2C). MOR and the
doses of 10 and 100 mg/kg of CBD significantly increased this latency (F_{4,48} = 26.1 p<0.01;
Figure 2C). CBD and MOR presented similar antinociceptive effects in this test (p>0.05;
Figure 2C). Antinociceptive effects in mechanical allodynia measured by Von Frey method
were observed after CXB, MOR and CBD (10 and 100 mg/kg) administration when compared
to CBD at 30 mg/kg (F_{3,32} = 26.2; p<0.05; Figure 2D).

### 3.3. Antinociceptive effects of chronic CBD on experimental parkinsonism

We also tested the antinociceptive effects of chronic treatment with CBD. Mice
received CBD (10 mg/kg i.p.) either in one single dose or daily dose for 14 days.

**Hyperalgesia response** – Both acute and chronic CBD increased hot plate response
latency (p<0.01) when compared to VEH group. The chronic CBD had significantly higher
antinociceptive effect compared to acute dose of CBD (F_{2,19} = 96.22; p<0.01; Figure 3A). In
tail flick, both acute and chronic CBD treatments significantly reduced this thermal
hyperalgesia when compared to VEH group (F_{2,19} = 14.26; p<0.01; Figure 3B). No
differences were found between acute and chronic CBD (p>0.05).

**Allodynia response** – The withdrawal latency for cold stimulus were increased in mice
that receive CBD, compared to VEH group (F_{2,19} = 8.881; p<0.01; Figure 3C). Similar effects
were found between acute and chronic treatment of CBD (p>0.05). The evaluation of
mechanical allodynia showed similar antinociceptive effects of both acute and chronic CBD
when compared to VEH (F_{2,19} =14.06; p<0.05; Figure 3D).

### 3.4. Antinociceptive effects of URB, AM251, Capsazepine and SCH 336 on
experimental parkinsonism

The progression in understanding the pain pathways have helped in discovering
several new pharmacological targets to treat pain: TRPV1 and the cannabinoid receptors
along with their endogenous agonists (endocannabinoids) are some of them.

**Hyperalgesia response** – Following three days of CBD washout, we tested the effect of URB, AM251, CPZ and SCH in lesioned-mice using the hot plate and tail flick tests. The drugs were administered 60 min previously to reach the plasmatic peak during the nociceptive tests (Deiana et al., 2012). There were no changes in the hot plate and tail flick responses latency in doses tested of VEH, URB, AM, CPZ and SCH in any of the groups (p>0.05; Figure 4A-B).

**Allodynia response** – URB, AM, CPZ and SCH effects were also tested in alldynia responses. There was no change in the withdrawal latency to a cold stimulus (Figure 4C) nor intensity of mechanical allodynia (Figure 4D) measured by Von Frey method in doses tested of VEH, URB, AM, CPZ and SCH (p>0.05).

### 3.5. Involvement of CB1, CB2 FAAH, and TRPV1 in the antinociceptive effects induced by CBD on experimental parkinsonism

To test the hypothesis that CBD could act on 6-OHDA-induced nociception via FAAH inhibition, and modulation of CB1, CB2 and TRPV1 receptors, we administrated CBD (30mg/kg) plus each drug related to the cited targets (URB, AM, CPZ and SCH). The hyperalgesia and alldynia responses were evaluated in mice with experimental parkinsonism. For this, the animals were randomly distributed into the groups after a new 3 days period of drug washout. The nociception tests were repeated before the experiment to ensure drug washout.

**Hyperalgesia response** – Pre-treatment with the FAAH inhibitor (URB) and TRPV1 antagonist (CPZ) potentiated the effect induced by CBD (interaction: F\(_{2,42}\)= 12.6; p<0.05, two-way ANOVA; p<0.05 vs. VEH and CBD; Figure 5A). On the other hand, pre-treatment with the CB1 receptor antagonist AM251 blocked the effects induced by CBD on hot plate response latency (interaction: F\(_{2,48}\)= 21.5; p<0.05, two-way ANOVA; p<0.05 vs. VEH and CBD). The CB2 inverse agonist SCH 336 had no influence on antinociceptive effects of CBD (p>0.05; Figure 5A). A similar finding was observed for tail flick latency. Pre-treatment with URB and CPZ potentiated the effect induced by CBD (interaction: F\(_{2,42}\)= 20.6; p<0.05, two-way ANOVA; p<0.05 vs. VEH and CBD; Figure 5B), while pre-treatment with AM and SCH blocked the effects induced by CBD on tail flick response latency (interaction: F\(_{2,48}\)= 21.5; p<0.05, two-way ANOVA; p<0.05 vs. VEH and CBD; Figure 5B).

**Allodynia response** – Administration of URB or CPZ potentiated the antinociceptive
effect of CBD (30 mg/kg) on withdrawal latency to a cold stimulus (interaction: $F_{2,50} = 6.2$; $p<0.05$, two-way ANOVA; $p<0.05$ vs. VEH and CBD; Figure 5C). Distinctly, pre-treatment with both AM and SCH blocked the effects induced by CBD in this test (interaction: $F_{2,48} = 8.7$; $p<0.05$, two-way ANOVA; $p<0.05$ vs. VEH and CBD; Figure 5C). Ineffective doses on allodynia of the URB or CPZ, when administered with an inefficacious dose of 30 mg/kg of CBD, evoked a synergistic increased in the withdrawal latency to a mechanical stimulus, suggesting similar mechanisms of action of the CBD, URB, and CPZ (interaction: $F_{2,48} = 18.1$; $p<0.05$, two-way ANOVA; $p<0.05$ vs. VEH and CBD; Figure 5D). On the other hand, AM and SCH did not change the lack of effect of the CBD in mechanical allodynia response ($p>0.05$; Figure 5D).

4. Discussion

The present results showed that: (i) experimental parkinsonism decreases the nociceptive threshold to thermal and mechanical stimulations, (ii) acute or chronic treatment with CBD decreases the hyperalgesia and allodynia in this condition, (iii) the highest CBD dose (100 mg/kg) induces a similar effect to morphine in parkinsonian mice, (iv) the inverse agonist of the CB1 receptor prevents the antinociceptive effect of CBD while ineffective doses of either a FAAH inhibitor or a TRPV1 antagonist increase the CBD antinociceptive effect. Altogether, these results indicate that acute or chronic treatment with CBD can be useful to reduce parkinsonism-increased nociception. They also suggest that CBD induces antinociception increasing endogenous anandamide levels and acting via CB1 and TRPV1 receptors, but not via CB2 receptors.

Our data showed an antinociceptive effect of the three analyzed doses of CBD in hyperalgesia responses (hot plate and tail flick tests). For allodynia responses, the lower and higher doses of CBD were significantly effective, whereas an intermediate dose did not work. Moreover, in three of the four performed tests (Figure 2B-D), a similar antinociceptive effect between CBD (100mg/kg) and morphine (MOR) was found. CBD was also compared to celecoxib (CXB), a nonsteroidal anti-inflammatory drug, which was minimally effective against both thermal and mechanical endpoints, which corroborates previous results (Hosseinzadeh et al., 2017). In our animal model, the highest dose of CBD promoted a better antinociceptive effect than CXB.

Hypernociception found in 6-OHDA lesioned animals are in line with several other studies in the field (Charles et al., 2018; Gee et al., 2015; Kaszuba et al., 2017; Zengin-Toktas et al., 2013). Hyperalgesia and allodynia can be mediated by central sensitization at distinct
central nervous system levels. At note, Charles and colleagues described a hyperexcitability of neurons from lamina V of the spinal cord in 6-OHDA-induced parkinsonism (Charles et al., 2018). The dorsal horn of the spinal cord is the first relay for nociceptive inputs from the periphery. Our data evidence decrease in nociceptive threshold in the first days after the 6-OHDA lesion when tail flick test was performed. Importantly, the tail flick use a noxious heat stimulation of the tail that produces a spinally mediated flexion reflex (Bannon and Malmberg, 2007). In contrast, in the hot-plate test, the final response involves not only spinal, but also supraspinal pathways.

Dennis and Melzack first described an essential role for the dopamine system in mediating antinociception (Dennis and Melzack, 1983). Basal ganglia, limbic areas, thalamus, periaqueductal gray matter (PAG), and other brain areas modulated by the dopaminergic system play a critical role in the processing of pain (Chudler and Dong, 1995; Hagelberg et al., 2002). The dopaminergic circuitry could also directly influence nociceptive behavior acting in the dorsal horn of spinal cord through hypothalamic A11 projections to spinal D2 receptors (Kim and Abdi, 2014; Taniguchi et al., 2011). Recent findings have shown a dysregulation of GABAergic neurotransmission in the PAG ventrolateral (the major component of the descending inhibitory pain pathway) induced by 6-OHDA nigrostriatal lesions, suggesting an impairment of descending analgesic system and stimulation of the descending pain facilitatory system (Domenici et al., 2019). This unbalance promotes downregulation of the spinal opioidergic modulation and, consequently, leads to a nociceptive sensitization. Based on these findings, we speculate that dysfunctions in limbic areas and at the spinal level could be responsible for the decreased nociceptive threshold observed in the 6-OHDA-induced PD model in the present work.

In patients, decreased function of the nigrostriatal pathway is associated with pain, (Hagelberg et al., 2004) while persistent and chronic pain reduces intracranial self-stimulation of the medial forebrain bundle (Leitl et al., 2014; Pereira Do Carmo et al., 2009). Since these pieces of evidence indicate that chronic pain leads to a significant impairment of dopamine activity (Chudler and Dong, 1995; Magnusson and Fisher, 2000; Taylor et al., 2016; Wood, 2008), the loss of dopaminergic nigrostriatal activation after injection of 6-OHDA into the striatum can directly contribute to the decreased nociceptive threshold observed in the current work.

The involvement of the endocannabinoid system in dopamine signaling within reward circuits affected by chronic pain is few explored (Mlost et al., 2019). Despite the antinociceptive potential of cannabinoids (Jensen et al., 2015), CBD effect on nociception
induced by striatal lesions in mice has never been investigated. Our results show that a CB1
receptor antagonist blunted CBD-induced antinociception while a CB2 receptor antagonist did
not affect CBD response, suggesting that CB1 receptors play an important role in CBD
antinociception. Although CBD has a low affinity for cannabinoid receptors, CBD can act
directly, at micromolar concentrations, as a partial agonist of CB1 and CB2 receptors
(McPartland et al., 2015; Pertwee, 2008a). CB2 receptors are mainly expressed in cells of the
immune system (Cabral and Griffin-Thomas, 2009) and mediated many anti-inflammatory
properties of cannabinoids (Turcotte et al., 2016), and albeit celecoxib has produced
antinociception in our experimental model of PD, CB2 receptors are unlikely to mediate CBD
effects in our study since the selective CB2 antagonist did not change the CBD
antinociception.

Conversely, a TRPV1 antagonist and a FAAH inhibitor increased the CBD-induced
analgesia, suggesting that the TRPV1 have a different role from CB1 increasing nociception
and that CBD actions might be depending on AEA. CBD can inhibit FAAH, increasing
endogenous AEA concentration (Leweke et al., 2012; McPartland et al., 2015). AEA is an
agonist of both CB1 and TRPV1 receptors (Di Marzo and De Petrocellis, 2012), as well as
can bind to CB2 receptor, but with less efficacy, behaving like a CB2 antagonist in some in
vitro bioassays (McPartland et al., 2015; Pertwee, 2008b). CB1 and TRPV1 have a distinct
mechanism of action: CB1 is a metabotropic receptor coupled to a Gi-protein while TRPV1 is
a channel permeable to several cations such as calcium (Di Marzo and De Petrocellis, 2012).
Therefore, CBD or AEA can produce opposite effects acting on these receptors (Di Marzo
and De Petrocellis, 2012).

Although the activation of CB1 receptors exert an anti-nociceptive and TRPV1
activation, a pro-nociceptive effect, the anandamide (AEA) has low intrinsic efficacy at
TRPV1 as compared to CB1 ((Ross, 2003; Toth et al., 2009; McPartland et al., 2015), and this
could explain the preference of the CB1, instead TRPV1, modulation by increased levels of
anandamide. In addition, the efficacy of AEA on TRPV1 is influenced by many factors and
physiological and pathological states, including the fact that AEA action on CB1 receptors
can change the TRPV1-mediated AEA response. In this sense, it is possible to suggest that the
smaller dose of CBD increase AEA, by inhibiting the FAAH, evoking a CB1-mediated
response while intermediate doses of CBD increase AEA to levels sufficient to activate both
CB1 and TRPV1 receptors eliciting lesser CBD-mediated antinociception, thus explaining the
U-shaped curve for the CBD in our experimental model. Another work using the FAAH
inhibitor URB597 injected into the PAG also found a dual effect for AEA in nociception. A
A low dose of URB597 into the ventrolateral PAG evoked a hyperalgesic effect that was converted to antinociceptive when injected along with the CB1 receptor inverse agonist AM251. However, a TRPV1 antagonist converted the antinociceptive effect of the high dose of URB597 to a hyperalgesic effect (Maione, 2006).

In vivo microdialysis showed that intraplantar injection of formalin increase AEA release within the PAG (Walker et al., 2002) while electrical stimulation of dorsal or lateral columns of the PAG evokes CB1-mediated antinociception along with a rise in AEA levels in the PAG (Martin et al., 1999). Moreover, mice lacking FAAH presents increased thermal analgesia and reduced nociceptive behavior in the formalin and carrageenan models (Carey et al., 2016). In the same experimental pain model, intra-spinal injection of AEA also evokes analgesia while CB1 receptor blockade inhibits these AEA effects (Carey et al., 2016). In the same way, the acute administration of URB597 decrease allodynia in a neuropathic pain model, and the pretreatment with a CB1 antagonist blunts this effect (Kinsey et al., 2009).

Altogether, these results suggest that AEA has antinociceptive effects acting via CB1 receptors in different experimental pain models.

The CB1 receptor is present in several brain regions involved in pain processing such as PAG, dorsal root ganglion, superficial laminae of the spinal cord, cortical areas, and amygdala (Di Marzo and De Petrocellis, 2012; Starowicz and Finn, 2017). Administration of WIN55,212-2, a synthetic cannabinoid agonist, into the amygdala increases tail-flick latency in rats, while the pharmacological blockade of CB1 receptors within the basolateral amygdala attenuates the stress-induced analgesia in the same pain mode (Connell et al., 2006). In nonhuman primates, the amygdala is essential to cannabinoid-induced antinociception (Manning et al., 2018), thus suggesting that amygdala may be a target for AEA-induced analgesia after peripheral CBD administration.

Amygdala and prefrontal cortex are areas that play a major role in mood disorders (Price and Drevets, 2010), and both are involved in the neurocircuitry of pain (Taylor, 2018; Xiao and Zhang, 2018). Neuroimaging studies show that these areas are affected in PD patients (Chagas et al., 2017; Yoshimura et al., 2005), thus contributing to the increased risk of anxiety and depression in these patients. Mood disorders can promote hypersensitivity to pain (Bushnell et al., 2013; Taylor, 2018), and a study showed a correlation between depression and pain in PD (Rana et al., 2017). Therefore, since several works demonstrate that CBD is useful to treat affective disorders such as depression (Blessing et al., 2015; Crippa et al., 2018; Campos et al., 2016), the modulation of the emotional component of pain can be part of CBD evoked-antinociception in our experimental model.
5. Conclusion

In summary, our results suggest that CBD decreases the enhanced nociception of animals with selective loss of dopaminergic nigrostriatal pathway. URB (selective FAAH inhibitor) treatment potentialized the CBD antinociception effect. On the other hand, the blockage of the CB1 receptors with the selective antagonist AM251 inhibited the CBD effect while TRPV1 receptor antagonism increased the antinociception effect of CBD. Altogether, these results suggest that CBD decrease the nociception inhibiting FAAH and consequently increasing AEA. In turns, AEA can bind to CB1 and TRPV1 receptors. These receptors have opposite effects on pain modulation. Thus, AEA binds to CB1 receptors promoting analgesia while decreases the nociception via TRPV1 receptors.

Acknowledgments

We thank Célia Aparecida da Silva for technical assistance and Mauricio dos Santos Pereira for drug offering.

Author contributions

G.C.N., D.P.F, M.B. and N.C.F.J. performed experiments. F.S.G. and E.A.D.B. provided feedback on project and participated in writing the manuscript. G.C.N., D.P.F, M.B. and N.C.F.J. designed study, analyzed data, interpreted results, and wrote the manuscript.

Financial Support

This study was supported by grants from CNPq, CAPES, and FAPESP.

Conflict interests

There is no conflict of interests to declare.

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**Figure legends**

Figure 1. Time-course of 6-OHDA-induced hyperalgesia and allosthenia responses. (A)
Photomicrographs of coronal sections from brains at different positions relative to Bregma illustrating the loss of TH+ fibers in the rostral (A'), medial (A'') and caudal (A''') striatum. 10x, Bar=50um. (B-D) Two-way analysis of variance (repeated measure) with Bonferroni’s post-hoc test for Basal measures (1 day before surgery: -1) and for 6-OHDA-treated rats 1, 7, 14 and 21 days after surgery. Decrease in thermal (Hot Plate - B, Tail Flick - C, Acetone-induced cold stimulus - D) and mechanical (Von Frey - E) withdrawal thresholds in 6-OHDA-treated rats have distinct temporal profiles. Data represent the mean ± SEM. *p < 0.05 from Basal (-1) measure, n = 8/test.

Figure 2. Antinociceptive effects of CBD on experimental parkinsonism. Antinociceptive effects induced by CBD (10, 30, and 100 mg/kg), in the Hot Plate (A), Tail Flick (B), acetone-induced cold stimulus (C), and Von Frey tests (D). Data represent the mean ± SEM. *p < 0.05 from VEH group, #p < 0.05 from CBD100 group; +p < 0.05 from MOR groups n = 8/group.

Figure 3. Antinociceptive effects of chronic therapy with CBD on experimental parkinsonism. Antinociceptive effects induced by chronic injection of CBD (10 mg/kg), in the Hot Plate (A), Tail Flick (B), acetone-induced cold stimulus (C), and Von Frey tests (D). Data represent the mean ± SEM. *p < 0.05 from VEH group, #p < 0.05 from CBD100 group; +p < 0.05 from MOR groups n = 8/group.

Figure 4. Antinociceptive effects of URB597 (URB), AM251 (AM), capsazepine (CPZ) and SCH336 (SCH) on experimental parkinsonism. Antinociceptive effects induced by URB (0.5 mg/kg), AM251 (1 mg/kg), CPZ (5 mg/kg) and SCH (2 mg/kg), in the Hot Plate (A), Tail Flick (B), acetone-induced cold stimulus (C), and Von Frey tests (D). Data represent the mean ± SEM. n = 8/group.

Figure 5. Involvement of CB1, CB2, FAAH and TRPV1 in the antinociceptive effects induced by CBD on experimental parkinsonism: proposal mechanisms. Effects of the pretreatment with FAAH inhibitor URB (0.5 mg/kg); CB1 receptor antagonist AM251 (1 mg/kg) and TRPV1 antagonist CPZ (5 mg/kg) followed by CBD (10 mg/kg) in the Hot Plate (A), Tail Flick (B), acetone-induced cold stimulus (C) and Von Frey tests (D). Data represent the mean ± SEM. *p < 0.05 from VEH group, #p < 0.05 from CBD10 group n = 5-7/group.
Highlights

• The CBD treatment decreases hyperalgesia and allodynia in experimental parkinsonism;

• The inverse agonist of the CB1 receptor prevents the antinociceptive effect of CBD;

• FAAH inhibitor or TRPV1 antagonist potentiates the CBD antinociceptive effect