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ORIGINAL ARTICLE

URB597 and the Cannabinoid WIN55,212-2 Reduce Behavioral and Neurochemical Deficits Induced by MPTP in Mice: Possible Role of Redox Modulation and NMDA Receptors

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Abstract Several physiological events in the brain are regulated by the endocannabinoid system (ECS). While synthetic cannabinoid receptor (CBr) agonists such as WIN55,212-2 act directly on CBr, agents like URB597, a fatty acid amide hydrolase (FAAH) inhibitor, induce a more "physiological" activation of CBr by increasing the endogenous levels of the endocannabinoid anandamide (AEA). Herein, we compared the pre- and post-treatment efficacy of URB597 and WIN55,212-2 on different endpoints evaluated in the toxic model produced by the mitochondrial toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice. MPTP (40 mg/kg, s.c., single injection) decreased locomotor activity, depleted the striatal and nigral levels of dopamine (DA), augmented the levels of lipid peroxidation and protein carbonylation in both regions, decreased the striatal protein levels of tyrosine hydroxylase, and increased the striatal protein content of the subunit 1 (NR1) of the *N*-methyl-D-aspartate receptor (NMDAr). Both URB597 (0.3 mg/kg, i.p., once a day) and WIN55,212-2 (10 μg/kg, i.p., twice a day), administered for five consecutive days, either before or after the MPTP injection, prevented the alterations elicited by MPTP and downregulated NMDAr. Our results support a modulatory role of the ECS on the toxic profile exerted by MPTP in mice via the stimulation of antioxidant activity and the induction of NMDAr downregulation and hypofunction, and favor the stimulation of CBr as an effective experimental therapeutic strategy.

Keywords MPTP toxic model · Oxidative stress · Neurochemical deficits · Endocannabinoid system · Cannabinoid receptor agonists · NMDAr hypofunction

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Introduction

Parkinson's disease (PD) is a neurodegenerative disorder first described in 1817 by James Parkinson (Goetz 2011). PD affects dopaminergic neurons from the substantia nigra pars compacta. At the clinical level, the disease is characterized by bradykinesia, muscular rigidity, postural instability, and tremor, affecting people with an average onset around 60 years of age (Kalia and Lang 2015). PD is conceptualized as a multifactorial disorder in which oxidative stress seems to be a causal event. Another triggering factor leading to cell damage in this disorder is mitochondrial dysfunction and excitotoxic processes induced by overactivation of *N*-methyl-D-aspartate (NMDA) receptors (NMDAr), further leading to increased levels of Ca^{2+}. Altogether, these processes lead to the activation of noxious signaling cascades and cell death (Schinder et al. 1996; Sgambato-Faure and Cenci 2012).

The mitochondrial toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a well-known complex I inhibitor, the effects of which were described in humans under accidental exposure (Langston et al. 1983). The parkinsonian syndrome produced by this agent in humans has served to establish an experimental model of PD (Burns et al. 1983; Langston et al. 1984; Smeyne and Jackson-Lewis 2005) that has been used in previous studies by our group (Garcia et al. 2014). MPTP crosses the blood-brain barrier and undergoes biotransformation into 1-methyl-4-phenylpyridinium (MPP^+) by monoamine oxidase B (MAO B) in astrocytes (Ransom et al. 1987), thus increasing the production of pro-inflammatory molecules such as TNF-α, IL-β, IL-6, iNOS, and nNOS (Mogi et al. 1998; Yang et al. 2009). Then, MPP^+ is transported into dopaminergic neurons by dopamine (DA) transporters (Chiba et al. 1985), hence increasing the production of free radicals (e.g., peroxynitrite, the main target of which is tyrosine hydroxylase, also known as TH) to produce cell dysfunction (Przedborski et al. 2000). MPTP-induced striatal and nigral alterations are accompanied by depletion of DA levels (Burns et al. 1983; Langston et al., 1983,1984; Garcia et al. 2014) and neurochemical deficits in dopaminergic neurons in the pars compacta (Perry et al. 1985a, 1985b) and their striatal terminals (Aguirre et al. 2005).

Toward the first formal description of the endocannabinoid system (ECS) (Self 1999), two types of G protein-coupled cannabinoid receptors (CBrs) have been characterized. In the brain, cannabinoid receptor 1 (CB1) is mainly localized in neuronal cells from the cortex, hippocampus, extrapyramidal and cerebellar areas, and basal nuclei (Elphick and Egertová 2001; Pagotto et al. 2006). In turn, cannabinoid receptor 2 (CB2) is known to be mainly located in microglia, the cells of the immune system in the brain (Cabral et al. 2008), although recently, some groups have demonstrated the expression of CB2 in neuronal, glial, and endothelial cells in the brain (Morales and Bonci 2012; Onaivi et al. 2012), whereby they could assist modulating immunological responses and motor functions. In particular, motor activity is modulated via interaction of dopaminergic D1 receptors with NMDAr in the striatum (thus involving the direct pathway), in which D1r directly interacts with the NR1 subunit to form a constitutive oligomeric complex which is recruited by the cell membrane in the presence of the NR2B subunit (Gardoni and Bellone 2015). In turn, the endogenous activation of all these receptors is mediated by endocannabinoids, such as anandamide (AEA), which are molecules derived from membrane phospholipids (Mechoulam and Fride 1995). Once synthesized from *N*-arachidonoyl-phosphatidyl-ethanolamine (NArPE) (Di Marzo 2011), AEA is released to the extracellular space to reach CBr and/or specific transporters located in neurons and astrocytes (Pacher et al. 2006). Indeed, dopaminergic D2 receptors have shown to be also involved in motor control through cannabinoid signals, since AEA is known to be released in the striatum upon D2 receptor stimulation in a metabotropic glutamate receptor-mediated mechanisms, thus contributing to long-term synaptic plasticity (Kreitzer and Malenka 2005). In the cytoplasmic domain, AEA is degraded by fatty acid amide hydrolase (FAAH), forming arachidonic acid and ethanolamine (Romero et al. 2002). It is noteworthy that some cannabinoid-related molecules can modulate metabolic processes of endocannabinoids at different levels. For instance, URB597 (formerly known as KDS-4103) is a compound derived from carbamate that blocks the FAAH activity through an irreversible carbamylation of the nucleophilic enzyme catalyzer for serine (Alexander and Cravatt 2005); therefore, URB597 is used as an endogenous pharmacological strategy to increase the endogenous levels of endocannabinoids (Russo et al. 2007). In rodents, URB597 reduces the brain hydrolysis of AEA, thus increasing its levels without altering the concentrations of 2-arachidonoylglycerol (2-AG) (Piomelli et al. 2006). In addition, a considerable number of synthetic cannabinoids have been produced; among them, WIN55,212-2 is known to act as an agonist for CB1 and CB2. Since WIN55,212-2 has been shown to reproduce the whole spectrum of in vivo effects produced by delta-9-tetrahydrocannabinol (Δ^9-THC) (Lauckner et al. 2005; Pertwee 2005), it represents a valuable pharmacological tool to explore the role of the ECS in experimental models of chronic disorders.

In this work, we compared the pre- and post-treatment effects of the ECS modulation by URB597 and WIN55,212-2 on different acute behavioral and neurochemical alterations induced by MPTP in the mouse brain. This study demonstrates modulatory effects of the tested cannabinoid agents at the regional (striatum vs. substantia nigra) and chronological (pre- vs. post-treatment) levels. Our results support the concept that the use of agents with the ability to modulate the ECS can ameliorate the deleterious actions of MPTP in a similar manner in the mice model of PD probably involving mechanisms oriented to counteract oxidative damage and downregulate NMDAr.

Materials and Methods

Reagents

MPTP hydrochloride, sodium octyl sulfate, sodium diethyldithiocarbamate trihydrate (DDC), HEPES, dithiothreitol, protease and phosphatase inhibitor cocktail, phenylmethylsulfonyl fluoride (PMSF), dopamine, WIN55,212-2, URB597, and the anti-actin (A5441) and anti-NR1 (G8913) antibodies were all purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were obtained from other commercial laboratories. Deionized water from a Milli-Q system (Millipore, MA) was used for preparation of solutions. The anti-TH (32-2100) antibody was from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). The goat anti-mouse IgG-HRP (sc-2005) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced

chemiluminescence (ECL) kit for Western blot was purchased from Amersham Life Sciences (Buckinghamshire, UK).

Animals

One hundred twenty C57BL/6NHsd male mice (8 weeks of age) weighing 25–30 g were used throughout the study. The animals were obtained from the vivarium of the Instituto Nacional de Neurología y Neurocirugía (INNN). Mice were housed five per cage, received a standard diet and water ad libitum, and synchronized with 12 h/12 h light-dark cycles under standard conditions of temperature (25 ± 1 °C) and 40% relative humidity. All procedures with mice were carried out strictly according to the local guidelines (Norma Oficial Mexicana NOM-062-ZOO-2001) for the use and care of laboratory animals as well as the "Guidelines for the Use of Animals in Neuroscience Research" from the Society of Neuroscience. The experiments were approved by the Ethics Committee of the INNN. All efforts were made to minimize animal suffering during the experiments. Minimal systemic toxicity, expressed as mice death, was observed after MPTP injection (only five mice died and were replaced by other animals).

Experimental Design

The study was carried out in two phases, testing two different treatment schemes (pre- and post-treatment) and using six groups per condition (n = 5–10 mice per group, depending on the experimental purpose). The CBr agonist WIN55,212-2 or the FAAH inhibitor URB597 was administered at two different times, prior to and after the exposure to MPTP, as described in the following sections. Both agents were prepared in a vehicle solution containing 5% Tween 80 and 5% DMSO in 0.9% saline solution. Each animal received WIN55,212-2 (10 μg/kg, i.p.) (modified from Chung et al. 2011) twice a day, or URB597 (0.3 mg/kg, i.p.) once a day, according to previous reports. In specific regard to the dose of URB597 used in this study, it has been demonstrated in in vivo studies that the dose chosen here produces an inhibitory effect on FAAH starting 15 min after its administration and lasting up to 12 h (Piomelli et al. 2006). MPTP was prepared in 0.9% saline solution and administered to mice as a single s.c. dose of 40 mg/kg, according to previous protocols (Perry et al. 1985a, 1985b; Aguirre et al. 2005; Palencia et al. 2015). Motor activity, the content of dopamine (DA), the levels of lipid peroxidation and protein carbonylation, and immunoblot assays were all estimated and performed in the striatum and substantia nigra of mice. For the analysis of samples from the substantia nigra, a pool (n = 5) was formed. This was due to the quantity of tissue obtained from the relatively small size of the region. The only exception to this consideration was the collection of nigral samples for immunoblot assays; for that case, enough protein from each individual sample was collected.

Experiment A: Pre-Treatment Protocol

Six different groups of mice were designed for this section: group A (control) received only vehicles; group B received two injections of WIN55,212-2 with a difference of 2 h between them during five consecutive days; group C received a single dose of URB597 also during five consecutive days; group D received a single dose of MPTP after receiving vehicle during 5 days; group E received WIN55,212-2 for 5 days prior to treatment with MPTP (the last cannabinoid administration was given 2 h after MPTP was injected); group F received URB597 for 5 days, prior to treatment with MPTP (the last URB597 administration was given 2 hours after MPTP was injected).

Experiment B: Post-Treatment Protocol

Similarly to the previous section, six different groups of mice were designed for this section: group A' (control) received only vehicles; group B' received two injections of WIN55,212-2 with a difference of 2 h between them during five consecutive days; group C' received a single dose of URB597 also during five consecutive days; group D' received a single dose of MPTP before receiving vehicle during 5 days; group E' received WIN55,212-2 for 5 days after being treated with MPTP (the first cannabinoid administration was given 2 h after MPTP was injected); group F' received URB597 for 5 days after treated with MPTP (the first URB597 administration was given 2 h after MPTP was injected).

The following endpoints were estimated at different times after either MPTP or the last cannabinoid administration, depending on their estimated occurrence in the toxic model. For instance, it is assumed that oxidative stress markers occur as an early and causal event during the progression of the toxic model, whereas motor and neurochemical markers could be expressions of cell and tissue dysfunction and require longer time courses to be expressed.

Motor Activity Assay: the Open Field Test

The behavioral study was carried out 6 days after the different treatments finalized, using an AccuScan device (AccuScan Instruments Inc., Columbus, OH) in a room with controlled conditions of light, temperature, and noise (Tatem et al. 2014). Mice were placed in the center of the device, and their motor activity was recorded for 15 min in 5 min intervals. The estimated parameters included the total distance walked (cm), the total number of horizontal and vertical movements, and the resting time (s).

Determination of Striatal and Nigral Dopamine Content

The total DA tissue content was analyzed by high-pressure liquid chromatography with electrochemical detection in samples of the striatum and substantia nigra, as previously described (Garcia et al. 1992). Animals (five per experimental group) were euthanized by cervical dislocation 7 days after MPTP (prophylactic protocol) or the last cannabinoid (therapeutic protocol) administration, assuming this time is appropriate to sense major neurochemical alterations. The brains were extracted, and the striatum and substantia nigra were dissected and immediately treated with 0.4 N perchloric acid and 0.1% (*w*/*v*) sodium metabisulphite, using 300 μl of this mixture for striatal samples. For the analysis of nigral samples, a pool ($n = 5$) was formed, using 200 μl of the buffer. All samples were homogenized and centrifuged (4000×*g*) at 4 °C during 10 min. The supernatants were filtered and kept at −70 °C until used for its HPLC analysis in a LC-4C Perkin-Elmer HPLC (Waltham, MA) with a BAS CC-5 electrochemical detector (West Lafayette, IN). Peaks were integrated with a Perkin-Elmer Turbochrom Navigator 4.1 data station. Calibration curves were constructed through the injection of standard solutions at known concentrations to the HPLC apparatus. The DA concentrations were obtained by interpolation of data with its respective pattern curve. An Alltech adsorbosphere catecholamine column (100 × 4.6 mm) with a particle size of 3 μm was used. The aqueous phase consisted of 0.1 M phosphate buffer (pH 3.1) containing 0.9 mM sodium sulfate, 0.1 mM EDTA, and 15% (*v*/*v*) methanol. The elution rate was fixed at 1.0 ml min^{-1}, whereas the potential was fixed at 0.8 V against an Ag/AgCl reference electrode. All samples were analyzed in duplicated.

Assay of Lipid Peroxidation

Lipid peroxidation was estimated in samples by the assay of thiobarbituric acid-reactive substances (TBA-RS), according to Buege and Aust (1978). Briefly, for this purpose, animals from different groups (five per experimental group) were euthanized by cervical dislocation 3 h after MPTP (prophylactic protocol) or the last cannabinoid (therapeutic protocol) administration, assuming early oxidative stress. The brains were extracted and the striatum and sustantia nigra were immediately dissected. Samples were kept in lysis buffer containing Tris base (1 mM) + sodium chloride (1.5 mM) + sucrose (0.02 mM). Protease inhibitors were used and included 1 mM PMSF, 1.7 mg/ml of aprotinin, 4 mM EDTA, and 0.05% of sodium azide. Three hundred microliters of the lysis buffer were used for the striatal samples, whereas 500 μl were used for the pool of nigral samples. After homogenization, 250 μl of the samples were separated into Eppendorf tubes and added with 500 μl of the TBA reagent containing 0.75 g of TBA + 15 g of trichloroacetic acid +2.54 ml of HCl. Samples were then incubated at 94 °C during 30 min in a shaking water bath. Immediately thereafter, samples were ice cooled for 5 min and centrifuged at 3000×*g* for 15 min. The optical density of the pink products was recorded in a Cytation 3 Cell Imaging Multi-Mode Reader (Biotek, Winooski, VT) at 532 nm. The levels of lipid peroxidation were calculated by interpolation with the values in a standard curve constructed and corrected by the protein content in each sample. Results were calculated as nanomoles of malonaldehyde (MDA) per milligram of protein and expressed as percent of increase vs. the control value.

Assay of Protein Carbonylation

For the estimation of the degree of protein carbonylation as another index of oxidative damage, animals from different groups (five per experimental group) were euthanized by cervical dislocation 3 h after MPTP (prophylactic protocol) or the last cannabinoid (therapeutic protocol) administration, assuming early oxidative stress as a cause of further major alterations. Immediately thereafter, striatal and nigral samples of mouse brains were homogenized in buffer solution and aliquots of 100 μl were collected in 1.5-ml Eppendorf probes to be centrifuged at 12000 rpm for 20 min. Protein carbonylation was calculated as an index of hydrazone formation after reaction with DNPH, as previously described (Colin-Gonzalez et al. 2013). The collected supernatants were incubated overnight with streptomycin sulfate (10%) to eliminate nucleic acids and re-centrifuged at 17,000×*g* (4 °C) for 20 min. The resulting supernatants were further treated with 10 mM DNPH (in 2.5 M HCl) for 1 h at room temperature. Then, TCA (10%) was added and samples were re-centrifuged at 2500×*g* (4 °C) for 10 min. The collected pellets were washed trice with ethanol/ethyl acetate (01:01) dissolved in 6 M guanidine chlorhydrate prepared in 20 mM phosphate buffer (pH 7.4). Samples were the centrifuged at 5000×*g* (4 °C) for 3 min to eliminate insoluble material. Optical density was estimated at 370 nm. The content of proteins containing carbonyl groups was expressed as nanomoles of DNPH per milligram of protein, using the molar absorption coefficient for DNPH (22.000 M–1 cm^{-1}). The total amount of protein was obtained through the estimation of the optical density at 280 nm in blank probes prepared in parallel (treated only with 2.5 M HCl), using a standard curve of BSA (0.25–2 mg/ml) prepared in 6 M guanidine chlorhydrate. Results were expressed as the percent of increase vs. the control value.

Immunoblot Assay

For this assay, animals (five per experimental group) were euthanized by cervical dislocation 10 days after MPTP (prophylactic protocol) or the last cannabinoid (therapeutic protocol) administration, assuming this time is optimum to find

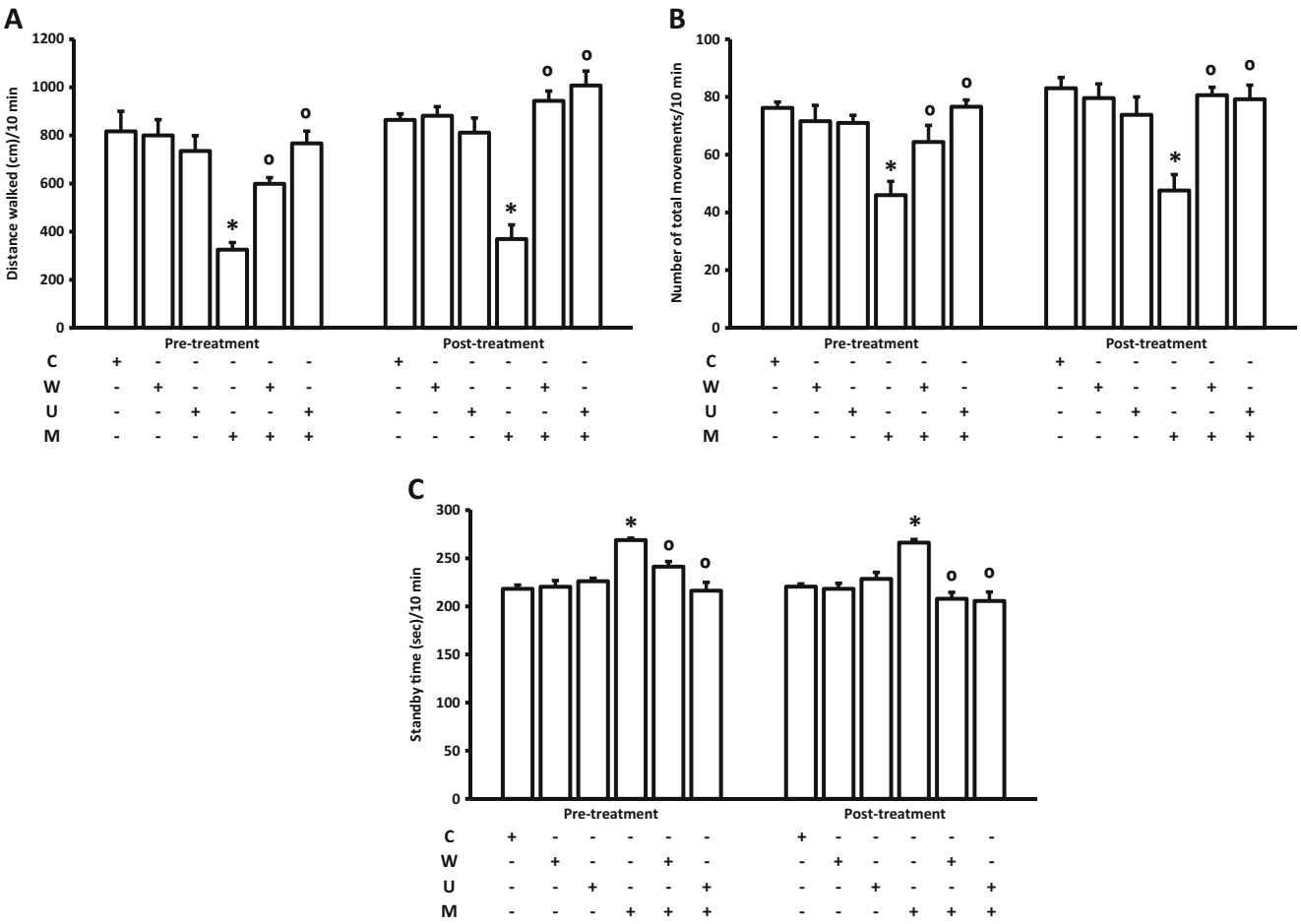

Fig. 1 Pre- and post-treatment effects of URB597 and WIN55,212-2 on MPTP-induced alterations in locomotor activity in mice. All animals received URB597 (0.3 mg/kg i.p. once a day × 5 days prior or after MPTP) or WIN55,212-2 (10 μg/kg, i.p. twice a day × 5 days prior or after MPTP) and MPTP (40 mg/kg, s.c., single dose). **a**, The total distance walked is presented. **b**, The total number of movements is shown. **c** The standby time is depicted. Mean values ± SEM of $n = 5$ mice per group are shown. *$p < 0.05$, difference of the control group; $^{0}p < 0.05$, difference of the MPTP group; two-way ANOVA followed by Bonferroni's test

major changes in proteins. The brains were extracted, and the striatum and substantia nigra were dissected and homogenized in lysis buffer also containing Triton (0.1%), PMFS (1 mM), aprotinin (1.7 mg/ml), EDTA (4 mM), and sodium azide (0.05%). For the specific case of membrane isolation for NR1 detection, the buffer also contained 250 mM sucrose and protease inhibitor cocktail (Calbiochem); then, membranes were obtained by successive centrifugations, strictly according to the method described by Ma et al. (2014). From these extracts, equal amounts of protein were calculated and used, following the Lowry's method (Lowry et al. 1951). Proteins were separated in polyacrylamide gel (10%) and transferred to nitrocellulose membranes. The membranes were blocked with a PBS solution + milk powder (5%) for 2 h at room temperature, to be further incubated overnight at 4 °C in the presence of the primary antibodies mouse anti-TH (Invitrogen, 32-2100, 1:1000, ~60 kDa) and anti-NMDA (anti-NR1, Sigma-Aldrich, G8913, 1:2000, ~106 kDa). Immediately thereafter, membranes were washed trice in PBS-Triton and incubated with secondary goat anti-mouse IgG-HRP antibody (Santa Cruz, sc-1468, 1:2000) for 1 h at room temperature. An enhanced chemiluminescence (ECL) kit for Western blot was used for bands detection and Kodak plates for developing. Immediately thereafter, each membrane was washed trice with PBS-Triton and stripped with a stripping buffer (100 mM 2-β mercaptoethanol + 2% SDS + 65.5 mM Tris HCl pH 6.8) in a shaking water bath at 50 °C for 25 min. Then, an antibody directed against β-Actin (Sigma-Aldrich, mouse IgG isotype A5441, 1:2000, ~42 kDa) was used for normalization of protein content. The ImageJ software was used for the densitometric analysis.

Statistical Analysis

Data are shown as mean values ± one standard deviation (SD), except for the behavioral data in which we expressed standard error medium (SEM). The statistical analysis was performed using the SPSS 21.0 software, using two-way ANOVA

followed by post hoc Bonferroni's test, with a minimum level of significance of $p \leq 0.05$.

Results

Locomotor Alterations Induced by MPTP to Mice Were Prevented by URB597 and WIN55,212-2

The open field test was used to compare the modulatory efficacy of URB597 and WIN 55,212-2, given prior or after the toxic insult, on MPTP-induced motor alterations in mice (Fig. 1). The optimum recording time was set at 10 min. Mice treated with MPTP displayed a hypoactive pattern of motor activity when compared with control animals that was evidenced by a decreased distance walked (60 to 57% below the control; Fig. 1a), a reduced number of movements (39 to 42% below the control; Fig. 1b), and an increased standby time (23 to 21% above the control; Fig. 1c).

Neither URB597 nor WIN55,212-2 exerted any change in the behavioral parameters evaluated. In contrast, when administered as pre-treatments to MPTP-treated mice, both agents prevented MPTP-induced decreased distance walked (84 and 136% above MPTP for WIN55,212-2 and URB597, respectively; Fig. 1a), the reduced number of movements (40 and 67% above MPTP for WIN55,212-2 and URB597, respectively; Fig. 1b), and the increased standby time (11 and 20% below MPTP for WIN55,212-2 and URB597, respectively; Fig. 1b). It is noteworthy that, when given to mice after the MPTP injection, these agents produced a more intense effect against MPTP-induced decreased distance walked (173 and 185% above MPTP for WIN55,212-2 and URB597, respectively; Fig. 1a), the decreased number of movements (75 and 72% above MPTP for WIN55,212-2 and URB597, respectively; Fig. 1b), and the increased standby time (23 and 25% below MPTP for WIN55,212-2 and URB597, respectively; Fig. 1c).

URB597 and WIN55,212-2 Ameliorated the MPTP-Induced Striatal and Nigral DA Depletion

The basal levels of DA in the striatum and the substantia nigra of control mice were 17.3 to 17.8 and 0.23 to 0.3 μg/g respectively (Fig. 2 and Table 1). The MPTP administration to mice decreased the striatal and nigral content of DA by ~75 and ~40% vs. control values, respectively. When tested alone, neither URB597 nor WIN55,212-2 produced changes in the striatal and nigral DA levels (Fig. 2 and Table 1).

As pre-treatment, WIN55,212-2 partially prevented the striatal and nigral MPTP-induced DA depletion (107 and 140% above MPTP, respectively; Fig. 2a and Table 1). When given after MPTP, WIN55,212-2 preserved the striatal and nigral levels of DA by 59 and 18% above MPTP, respectively (Fig. 2b and Table 1).

Either given prior or after the MPTP administration to mice, URB597 recovered the striatal MPTP-induced DA depletion by 221 and 90% above MPTP, respectively (Fig. 2a, b). It also preserved the nigral DA levels by 70 and 31% above MPTP, respectively (Table 1).

URB597 and WIN55,212-2 Decreased MPTP-Induced Oxidative Damage to Lipids

Basal levels of lipid peroxidation in striatal and nigral tissues of control mice were normalized to 100%. The levels of lipid peroxidation (expressed as the percent of TBA-reactive substances vs. control) induced by MPTP were increased by 38

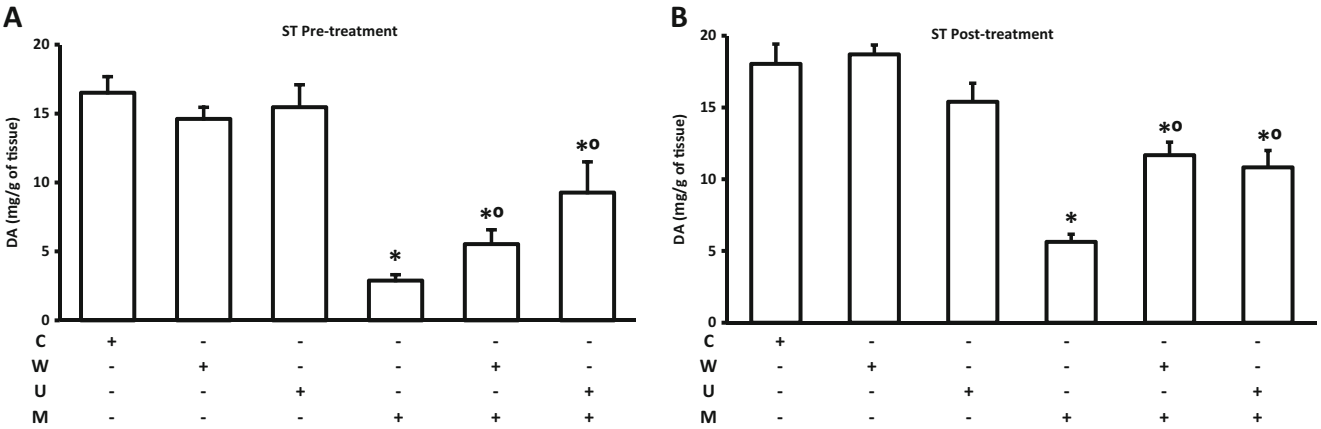

Fig. 2 Pre- and post-treatment effects of URB597 and WIN55,212-2 on MPTP-induced changes in brain dopamine (DA) levels in mice. All animals received URB597 (0.3 mg/kg i.p. once a day × 5 days prior or after MPTP) or WIN55,212-2 (10 μg/kg, i.p. twice a day × 5 days prior or after MPTP) and MPTP (40 mg/kg, s.c., single dose). **a**, **b** The striatal levels of DA when treatments were given before and after MPTP injection. Mean values ± SEM of $n = 5$ mice per group are shown for striatal determinations. $^{*}p < 0.05$, difference of the control group; $^{0}p < 0.05$, difference of the MPTP group; two-way ANOVA followed by Bonferroni's test

Table 1 Effects of WIN55,212-2 and URB597 as pre- or post-treatments on dopamine, lipid peroxidation, and carbonyl levels in the substantia nigra of MPTP-treated mice

Biochemical endpoints in SN pre-treatment			
Group	% Dopamine	% TBA-reactive substances	% Carbonyl groups
Control	100	100	100
WIN55,212-2	83.21	69.3	94.18
URB597	92.70	141.8	119.5
MPTP	44.93	203.5	184.8
WIN55,212 + MPTP	104.62	62.28	87.83
URB597 + MPTP	72.70	124.1	113
Biochemical endpoints in SN post-treatment			
Group	% Dopamine	% TBA-reactive substances	% Carbonyl groups
Control	100	100	100
WIN55,212	93.59	88.1	140.2
URB597	94.70	104.1	172.1
MPTP	74.78	203.5	184.8
WIN55,212-2 + MPTP	85.85	64.29	103.3
URB597 + MPTP	121.07	109.6	84.8

and 104% in the striatum and nigra, respectively (Fig. 3 and Table 1).

WIN55,212-2 administration, given as a pre-treatment, prevented MPTP-induced lipid peroxidation in the striatum and the nigra (43 and 70% below MPTP, respectively; Fig. 3a and Table 1). The same antioxidant effect was achieved in the striatum and nigra when WIN55,212-2 was given after MPTP injection (36 and 68% below MPTP, respectively; Fig. 3b and Table 1).

As pre-treatment, URB597 prevented the levels of lipid peroxidation induced by MPTP in the striatum and nigra (23 and 39% below MPTP, respectively; Fig. 3a and Table 1). In contrast, when administered after MPTP injection, as therapeutic, it did not reduce the oxidative damage induced by MPTP in the striatum (3% below MPTP; Fig. 3b), although in the nigra, this agent produced a reduction of oxidative damage to lipids (47% below MPTP; Table 1).

URB597 and WIN55,212-2 Decreased MPTP-Induced Levels of Protein Carbonylation

Basal levels of protein carbonylation in the striatum and nigra in control mice were normalized to 100%. The content of protein carbonylation (represented as percent above the control) induced by MPTP was found increased by 32 and 85% in the striatum and nigra, respectively (Fig. 4 and Table 1).

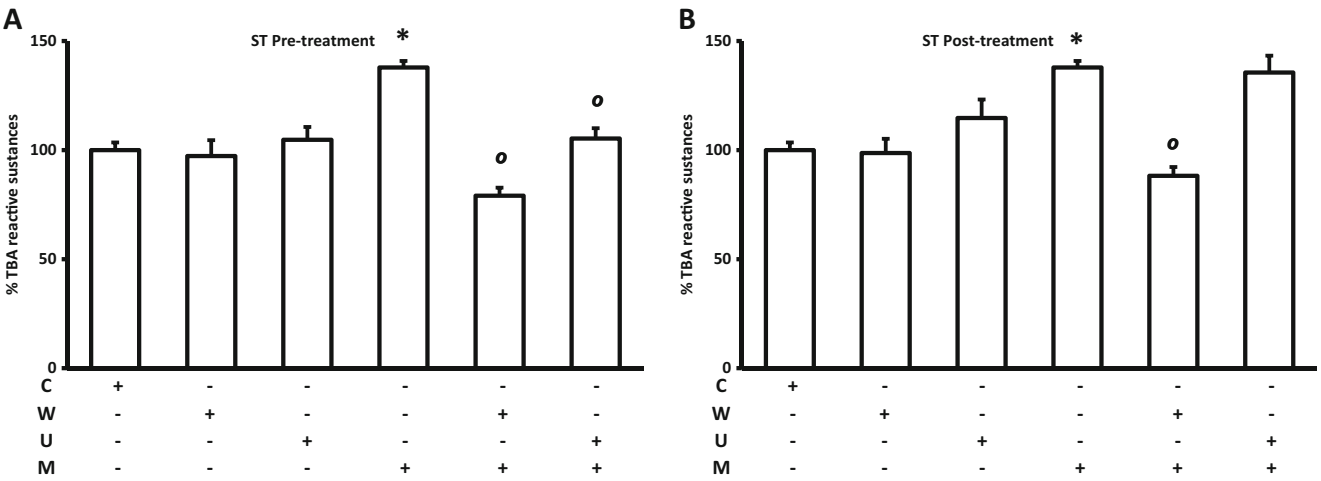

Fig. 3 Pre- and post-treatment effects of URB597 and WIN55,212-2 on MPTP-induced brain lipid peroxidation in mice. All animals received URB597 (0.3 mg/kg i.p. once a day × 5 days prior or after MPTP) or WIN55,212-2 (10 μg/kg, i.p. twice a day × 5 days prior or after MPTP) and MPTP (40 mg/kg, s.c., single dose). **a**, **b** The striatal levels of TBA-reactive substances when treatments were given before and after MPTP injection. Mean values ± SEM of $n = 5$ mice per group are shown for striatal determinations. $^{*}p < 0.05$, difference of the control group; $^{0}p < 0.05$, difference of the MPTP group; two-way ANOVA followed by Bonferroni's test

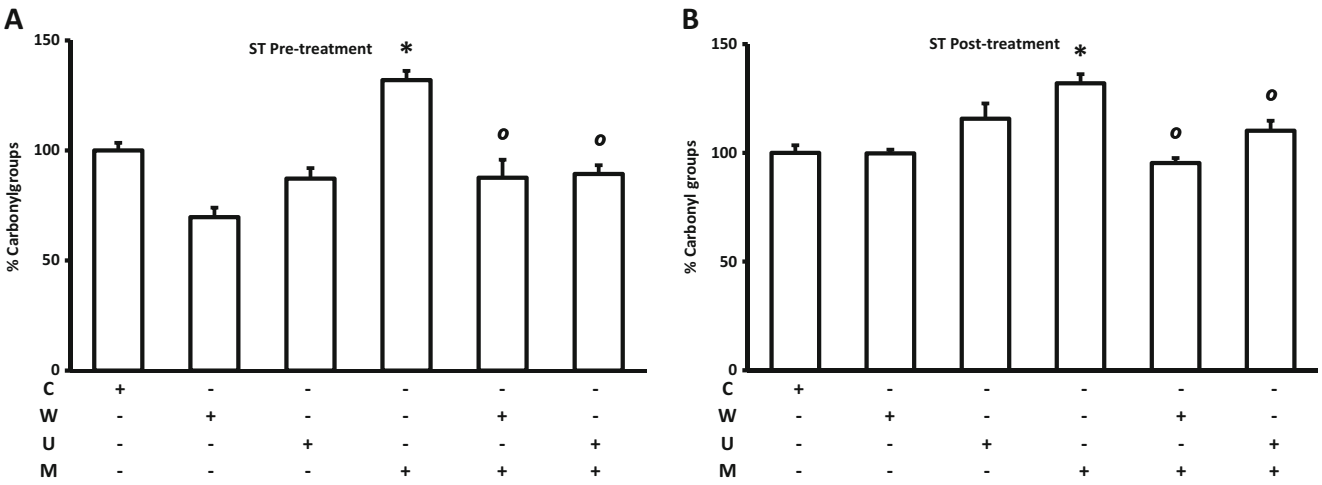

Fig. 4 Pre- and post-treatment effects of URB597 and WIN55,212-2 on MPTP-induced brain protein carbonylation in mice. All animals received URB597 (0.3 mg/kg i.p. once a day × 5 days prior or after MPTP) or WIN55,212-2 (10 μg/kg, i.p. twice a day × 5 days prior or after MPTP) and MPTP (40 mg/kg, s.c., single dose). **a**, **b** The striatal levels of carbonyl groups when treatments were given before and after MPTP injection. Mean values ± SEM of $n = 5$ mice per group are shown for striatal determinations. $^{*}p < 0.05$, difference of the control group; $^{0}p < 0.05$, difference of the MPTP group; two-way ANOVA followed by Bonferroni's test

WIN55,212-2 decreased the MPTP-induced carbonylation in the striatum and nigra when given as pre-treatment (34 and 53% below MPTP, respectively; Fig. 4a and Table 1). Similarly, the amounts of protein carbonylation induced by MPTP in the striatum and nigra were decreased by WIN55,212-2 when given after the toxin injection (28 and 44% below MPTP, respectively; Fig. 4b and Table 1).

As pre-treatment, URB597 decreased the levels of protein carbonylation induced by MPTP in the striatum and nigra (33 and 39% below MPTP, respectively; Fig. 4a and Table 1). When administered after MPTP, URB597 also decreased the levels of oxidative damage to proteins in the striatum and nigra (17 and 54% below MPTP, respectively; Fig. 4b and Table 1).

URB597 and WIN55,212-2 Preserved the Expression Levels of TH and NR1 in the Striatum and Substantia Nigra of Mice Exposed to MPTP

Figure 5 depicts the effects of URB597 or WIN55,212-2 on MPTP-induced striatal and nigral downregulation of TH and upregulation of NR1 in mice. MPTP administration significantly decreased the protein levels of TH in the striatum and nigra (30 and 36% below the control, respectively; Fig. 5a, c). The administration of WIN55,212-2 to MPTP-treated mice preserved the levels of TH in both schemes, pre- and post-treatment, in the striatum (74 and 82% above MPTP, respectively; Fig. 5a) and nigra (98 and 84% above MPTP, respectively; Fig. 5c).

In turn, URB597 preserved the levels of TH in both schemes of administration, pre- and post-treatment, in the striatum (65 and 96% above MPTP, respectively; Fig. 5a) and nigra (58 and 100% above MPTP, respectively; Fig. 5c).

In regard to NR1 expression, MPTP induced an upregulation in the striatum (88% above the control; Fig. 5b) and nigra (34% above the control; Fig. 5d). The administration of WIN55,212-2 to MPTP-treated mice decreased the protein expression in both schemes of administration, pre- and post-treatment, in the striatum (47 and 51% below MPTP, respectively; Fig. 5b) and nigra (46 and 36% below MPTP, respectively; Fig. 5d). When URB597 was administered to MPTP-treated mice, the NR1 expression levels were decreased in both schemes of administration, pre- and post-treatment, in the striatum (46 and 52% below MPTP, respectively; Fig. 5b) and nigra (54 and 50% below MPTP, respectively; Fig. 5d).

Finally, the administration of both agents alone (WIN55,212-2 and URB597) to mice did not alter the expression of TH, but as expected, they significantly reduced the levels of NR1 in the nigra.

Discussion

In this study, we compared for the first time the modulatory efficacy of two agents described to exert regulatory effects on the ECS through different mechanisms, when administered as pre- and post-treatments to MPTP. We investigated the effects of a direct activation of CB1 by WIN55,212-2 vs. the potential induction of AEA accumulation by URB597, on different toxic endpoints estimated in the model induced by MPTP in mice. Particular emphasis was given to neurochemical deficits and oxidative damage. Both agents exhibited similar patterns of preventive and modulatory properties, suggesting that the efficacy of both agents is related with the same mechanism recruited: activation of CBr and reduction of neuronal

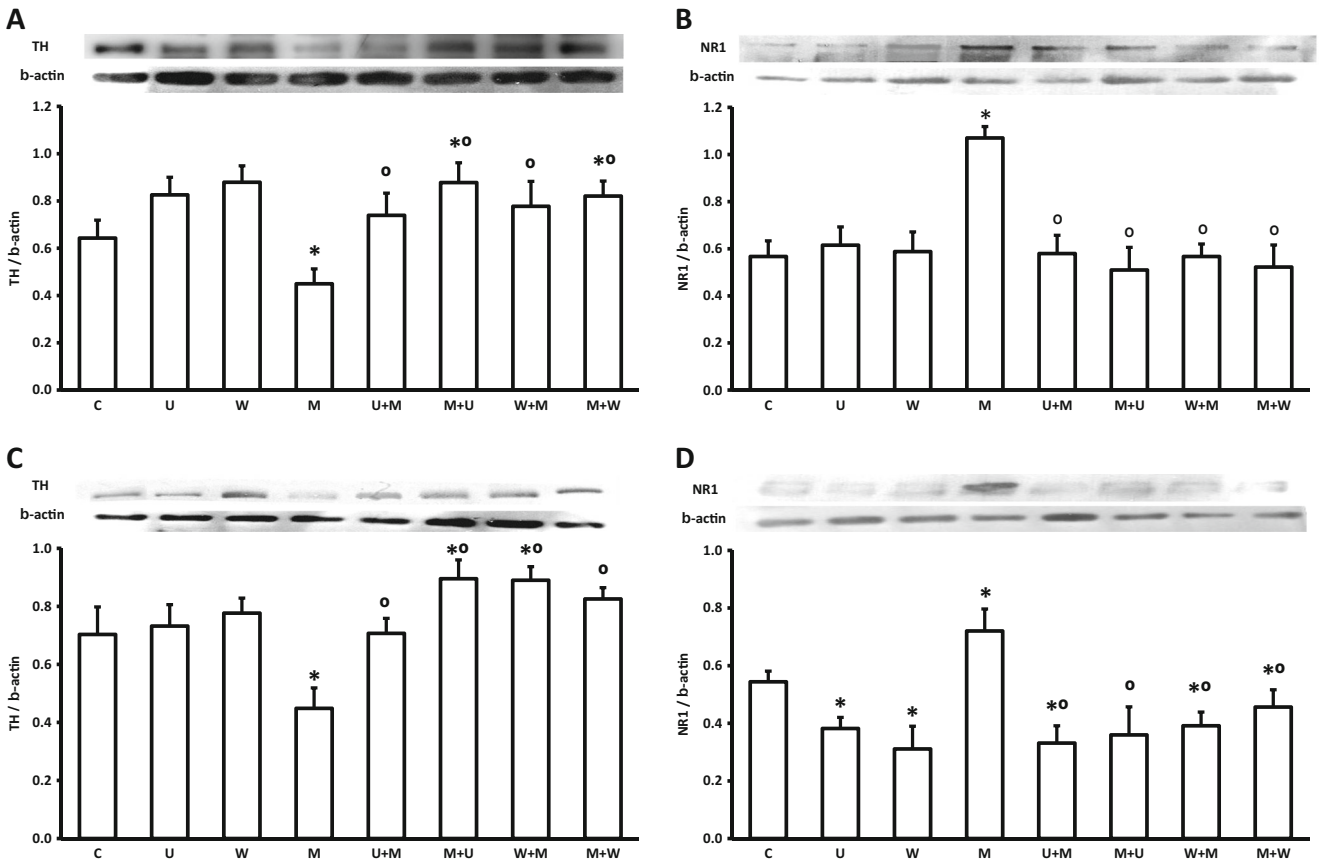

Fig. 5 Pre- and post-treatment effects of URB597 and WIN55,212-2 on MPTP-induced changes in the brain protein levels of tyrosine hydroxylase (TH) and NR1 subunit of NMDA receptors in mice. All animals received URB597 (0.3 mg/kg i.p. once a day × 5 days prior or after MPTP) or WIN55,212-2 (10 μg/kg, i.p. twice a day × 5 days prior or after MPTP) and MPTP (40 mg/kg, s.c., single dose). **a**, **c** The striatal and nigral levels of TH when treatments were given before and after MPTP injection. **b**, **d** The striatal and nigral levels of NR1 when treatments were given before and after MPTP injection. Mean values ± SEM of $n = 5$ mice per group are shown for striatal and nigral determinations. For all panels, W+M and U+M denote pretreatments, whereas M+W and M+U denote post-treatments. $^{*}p < 0.05$, difference of the control group; $^{0}p < 0.05$, difference of the MPTP group; two-way ANOVA followed by Bonferroni's test

alterations by decreased oxidative damage and excitability through NMDAr downregulation. Most importantly, both agents were similarly able to exert these modulatory effects both as pre-treatments and as post-treatments. Both URB597 and WIN55,212-2 supported the concept that pharmacologically induced accumulation of endogenous cannabinoids as well as direct stimulation of CBr constitute physiological and effective modulatory strategies against MPTP-induced behavioral and neurochemical alterations. In fact, these strategies have also shown to minimize undesired effects, such as the psychoactive features accompanying the direct activation of CB1 through synthetic CBr agonists (Romero et al. 2002).

Both URB597 and WIN55,212-2 were effective in preventing motor alterations in MPTP-treated mice, including the total distance walked, the total number of movements, and the standby time, when given to mice as pre-treatments. It is noteworthy that both agents were also equally effective to prevent mice from these alterations when administered after MPTP injection. The same scenario occurred for the striatal levels of DA, in which URB597 resulted similarly effective than WIN55,212-2 to prevent DA depletion when these agents were administered as pre-treatments. Once again, both agents were equally effective to prevent the striatum against the MPTP-mediated loss of this neurochemical endpoint when administered as post-treatments. Only WIN55,212-2 was effective to completely prevent DA loss in the substantia nigra when administered as a pre-treatment, although URB597 partially preserved DA levels. Interestingly, when given as post-treatments, both agents almost completely preserved nigral DA levels.

One of the fewest markers in which WIN55,212-2 exhibited a better preventive profile than URB597 was lipid peroxidation. Uncoupling of mitochondrial complex I in SNpc is known to increase lipid peroxidation in the nigrostriatal system, leading to neuronal cell damage. It has been hypothesized that axonal vulnerability to oxidative stress is due to the high content of DA in the nigrostriatal system. When neuronal cell bodies are damaged in the SNpc, there is a considerable release of DA to the extracellular space, where this neurotransmitter suffers free radical attacks, mainly by hydroxyl radical

and auto-oxidation, then favoring oxidative damage to lipids (Burke and O'Malley 2013; Obata 2002). In addition, free MPP^+ at the extracellular space is actively re-uptaken by terminal axons at the striatum, in which retrograde transport and the high concentration of polyunsaturated lipids could facilitate oxidative stress (Hung and Lee 1998). As pre-treatment, WIN55,212-2 reduced this marker in the striatum and nigra even below the basal levels, whereas as post-treatment, in contrast to URB597, it was able to preserve the redox integrity of the striatum. This effect could be due to inner antioxidant properties of WIN55,212-2, which might act not only as CBr agonist but also as a free radical scavenger. In turn, oxidative damage to proteins was equally prevented by both agents in the striatum and nigra. We could also hypothesize that URB597 is acting as an antioxidant, as evidenced by its effects in the carbonylation test, as well as the lipoperoxidation test when given as pre-treatment. However, the lack of effect of this agent on lipid peroxidation when given as a post-treatment reveals that its pharmacological potency against this endpoint was not sufficient to counteract the ongoing actions of ROS.

Probably one of the most relevant and novel findings of our study is the link between the TH and the NR1 protein regulation induced by URB597 and WIN55,212-2 in the striatum and nigra of MPTP-treated mice. While TH was decreased by MPTP in both regions, and both cannabinoid agents prevented and preserved this neurochemical marker, the fact that NR1 was upregulated by MPTP in both brain regions, and the two cannabinoid agents were able to prevent this upregulation, suggest, at least in a preliminary manner, that a glutamatergic component implicating overactivation of NMDAr could be involved in the toxic pattern elicited by MPTP in the brain. Congruent with this concept, the use of cannabinoid agents with the ability of activating CB1 and the subsequent triggering of a mechanism that abducts NR1 for its degradation—therefore inducing NMDA hypofunction and further prevention of excitotoxicity (Sánchez-Blázquez et al. 2014)—constitute a plausible explanation to contribute the modulatory actions observed in this paradigm. This consideration is supported by the effect of both agents per se on NR1, since this protein was downregulated in the nigral tissue, suggesting a long-term tendency to produce glutamatergic hypofunction that remains to be demonstrated in further studies.

The issue of a role of the ECS modulation on MPTP-induced toxicity in animal models is not new. Different research groups have addressed this topic previously. For instance, in 2001, Lastres-Becker et al. (2001) studied the CB1 binding and the degree of stimulation by WIN55,212-2 of [^{35}S]GTPγS binding to membrane fractions in the caudate nucleus, putamen, lateral globus pallidus, and substantia nigra of brains from PD patients. These authors found that the enhanced binding revealed an effective GTP-binding protein-coupled signaling mechanism via CB1 and an increased CB1 binding in the caudate nucleus and the putamen. They confirmed these findings in MPTP-lesioned marmosets. These animals exhibited an increased CB1 binding and stimulation of [^{35}S]GTPγS binding by WIN55,212-2 in the caudate nucleus and putamen, compared to control animals, but this tendency was reversed by L-dopa therapy. The conclusion derived from these experiments was that the blockade of CB1 might contribute to the treatment of parkinsonian motor symptoms. Although these findings might initially point against a protective role of the ECS upregulation in PD and in the MPTP model, in first instance, they could suggest that CB1 upregulation might represent a compensatory response of the basal ganglia to counteract the ongoing damaging events. Since we did not define which receptor mediates protection in the model tested in this study, reports like the abovementioned, or the discussed forward, are enlightening when inferring this issue.

In contrast to Lastres-Becker's report, in 2009, Price et al. (2009) investigated the molecular mechanisms by which WIN55,212-2, given up to 24 h after the injection of MPTP to mice, protected the nigrostriatal pathway. By stereological means, these authors found that the cannabinoid agent was able to prevent the loss of TH neurons in the substantia nigra and the striatal and nigral loss of DA in a mechanism apparently independent of CB1 activation. Interestingly, they found that the neuroprotective action of WIN55,212-2 could be more related with the CB2 activation and further microglial regulation, as well as that chronic treatment with the cannabinoid also reversed the motor deficits induced by MPTP in mice, then supporting a protective role of this agent through the ECS modulation in the MPTP toxic model at different levels (behavioral, biochemical, cellular, and molecular). Despite their results were obtained in a different model of MPTP toxicity, and different endpoints were estimated (including a behavioral motor approach), positive actions of this CBr agonist were obtained by them and us in our respective protocols, thus emphasizing the therapeutic potential of this pharmacological tool. The same group in the same year (Morgese et al. 2009) investigated the neurochemical changes underlying the anti-dyskinetic effect of WIN55,212-2 in a PD model produced by 6-hydroxydopamine in rats using in vivo microdialysis in the dorsal striatum. These authors reported that dyskinesia was related with the L-dopa-induced loss of extracellular glutamate and a disruption in the glutamatergic/dopaminergic transmission, and the subchronic administration of the cannabinoid agent prevented this neurochemical effect. Although their findings bring partial explanation about the modulatory action of WIN55,212-2 on the dyskinetic pattern displayed in PD models through DA and glutamate outputs, it does not provide full integration on the whole preventive role of the ECS modulation in PD and related animal models.

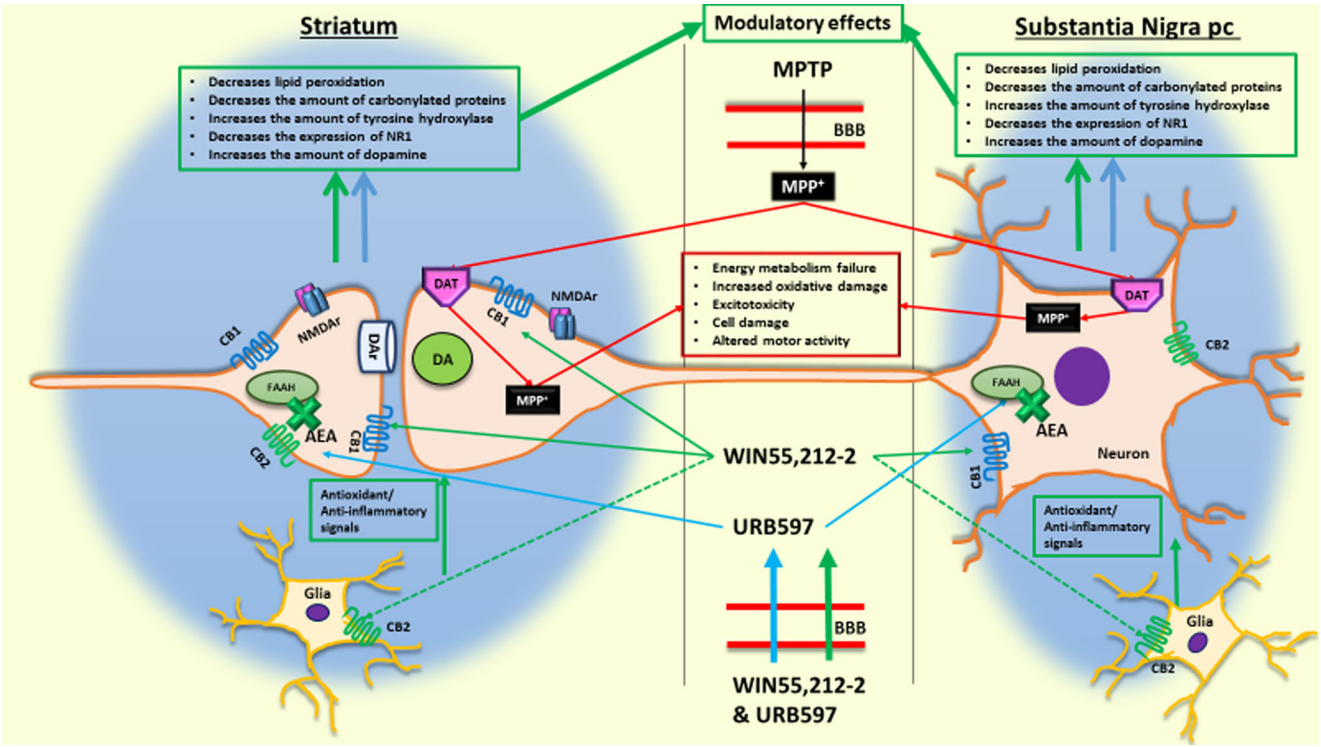

Fig. 6 Schematic representation of the proposed mechanisms occurring in the toxic model induced by MPTP and the modulatory actions exerted by the synthetic cannabinoid agonist WIN55,212-2 and the FAAH inhibitor URB597 in the mice striatum and substantia nigra. MPTP crosses the blood-brain barrier (BBB) and is converted into MPP^+ by glial cells. MPP^+ then is internalized in dopaminergic synaptic terminals in the striatum, or in dopaminergic cells in the substantia nigra through dopamine transporters (DAT), from which it can trigger toxic events, including mitochondrial dysfunction and energy depletion, reactive oxygen species (ROS) formation, and oxidative stress, excitotoxicity, and activation of toxic cascades, ultimately leading to behavioral and neurochemical deficits in the nigrostriatal pathway. The toxic events elicited by MPTP are marked in *red*. In turn, WIN55,212-2 also crosses the BBB to reach CB1 located on the vicinity of NMDAr or dopamine receptors (DAr) in pre- or post-synaptic endings in the striatum or in DAergic neurons in the substantia nigra. The activation of CB1 by WIN55,212-2 could modulate glutamatergic and/or DAergic transmission, thereby reducing excessive oxidative and excitatory activities. In addition, WIN55,212-2 could also act on CB2 located in microglia, hence modulating immunological events and promoting modulatory responses, preventing the MPTP-induced striatal and nigral toxicity. The signals elicited by WIN55,212-2 are marked in *green*. Finally, URB597 also crosses the BBB and reaches the striatal and nigral compartments, in which it binds FAAH, the enzyme responsible for degradation of AEA, therefore inducing an accumulation of this endocannabinoid, which in turn will activate CB1 and/or CB2 to promote modulatory effects on MPTP-induced toxicity at the synaptic and somatic levels. The signals elicited by URB597 are marked in *blue* (color figure online)

More recently, the specific preventive role of CB1 and a more prominent role of the ECS activation on different markers of inflammation, oxidative stress, and neurodegeneration, in the mouse model of PD produced by MPTP, were described (Chung et al. 2011). Through the use of the non-selective CB1 agonists WIN55,212-2 and HU210, as well as the selective CB1 antagonists AM251 and SR14,716A, it was suggested that the CB1 activation inhibits microglial activation and oxidative stress and concluded that the ECS is beneficial for treatment of PD and experimental models. Our findings are more in agreement with this study, although the dyskinetic component associated to WIN55,212-2 treatment remains unsolved.

To our knowledge, the use of the specific inhibitor of FAAH, URB597, to induce AEA accumulation as a modulatory strategy in PD models has only been reported in a couple of previous reports. Johnston and coworkers (Johnston et al. 2011) explored the effect of URB597 on the dyskinetic behavior exhibited by MPTP-treated marmosets also receiving L-dopa. Therefore, the particular emphasis of their study was focused on the alleviating action of ECS modulator on dyskinesia. Of note, URB597 reduced these motor alterations without affecting the protective actions of L-dopa. More recently, Celorrio et al. (2016) addressed the issue of a chronic inhibition of FAAH by URB597 in the parkinsonian model induced by MPTP + probenecid (MPTPp) administration over 5 weeks. These authors reported that URB597 was able to prevent the MPTPp-induced motor impairment, but it did not preserve DA levels in the nigrostriatal pathway or regulate cell activation. In this regard, while this evidence supports FAAH inhibition as a strategy to control motor disturbances of Parkinson-like alterations, it contrasts with our results demonstrating a modulatory effect of

URB597 also on neurochemical markers. Thus, differences in the effects obtained with this agent deserve further and detailed investigation in both chronic and acute models of MPTP toxicity. In summary, our study supports the concept of a modulatory role of this agent oriented to its potential to induce AEA accumulation and demonstrates for the first time that the use of URB597 as pre- and post-treatment in the acute MPTP mouse model constitutes a valuable tool for further experimental studies and the design of FAAH inhibitor-based drugs for the treatment of different forms of parkinsonism.

Of final consideration for the modulatory role of URB597 in PD, Kreitzer and Malenka (2007) described an effect of AEA in the regulation of long-term depression (LTD) through dopamine D2 receptor activation in the indirect pathway, which in turn is crucial for controlling motor activity. LTD is absent in models of PD, but it can be rescued, together with motor deficits, by D2 receptor agonists and/or inhibitors on endocannabinoid degradation such as URB597. This evidence emphasizes the relevance of pharmacological manipulation of the cannabinoid system for modulatory purposes in PD and other neurodegenerative disorders.

Conclusions

The stimulation of the ECS in the brain either through the direct action of CBr agonists or by agents inducing the accumulation of endocannabinoids constitutes a valuable therapeutic strategy against oxidative events as well as neurochemical and behavioral deficits produced in the brain by different toxic insults. The finding that both URB597 and WIN55,212-2 exhibited similar modulatory profiles on some behavioral and neurochemical endpoints of toxicity elicited by MPTP strengthens our perception that the ECS stimulation has an enormous potential to design pharmacological tools through similar mechanisms based on cannabinoid drugs, as it can preserve the brain functions even after the toxic cascades have been initiated. Finally, the suggested mechanisms by which both URB597 and WIN55,212-2 are producing modulatory effects on the toxic events elicited by MPTP in the corpus striatum and substantia nigra of mice are summarized in Fig. 6.

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Compliance with Ethical Standards All procedures with mice were carried out strictly according to the local guidelines (Norma Oficial Mexicana NOM-062-ZOO-2001) for the use and care of laboratory animals as well as the "Guidelines for the Use of Animals in Neuroscience Research" from the Society of Neuroscience. The experiments were approved by the Ethics Committee of the INNN.

Conflict of Interest The authors declare that they have no conflict of interest.

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