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WIN55,212-2, a Cannabinoid Receptor Agonist, Protects Against Nigrostriatal Cell Loss in the MPTP Mouse Model of Parkinson's Disease

David A. Price^{1,*}, Alex A. Martinez¹, Alexandre Seillier¹, Wouter Koek^{1,2}, Yolanda Acosta¹, Elizabeth Fernandez^{1,3}, John R. Strong^{1,3}, Beat Lutz⁴, Giovanni Marsicano⁵, James L. Roberts^{1,6}, and Andrea Giuffrida¹

¹ Department of Pharmacology, University of Texas Health Science Center, San Antonio, Texas

² Department of Psychiatry, University of Texas Health Science Center, San Antonio, Texas

³ Geriatric Research, Education and Clinical Center, South Texas Veterans Health Care System, San Antonio, Texas

⁴ Institute of Physiological Chemistry and Pathobiochemistry, Johannes Gutenberg-University Mainz, Germany

⁵ NeuroCentre Magendie, U682 INSERM, University of Bordeaux 2, Avenir Group, Bordeaux, France

⁶ Audie L. Murphy Veteran Affairs Medical Center, San Antonio, Texas

Abstract

Parkinson's disease (PD) is characterized by the progressive loss of nigrostriatal dopamine (DA) neurons leading to motor disturbances and cognitive impairment. Current pharmacotherapies relieve PD symptoms temporarily but fail to prevent or slow down the disease progression. In this study, we investigated the molecular mechanisms by which the non-selective cannabinoid receptor agonist WIN55,212-2 (WIN) protects mouse nigrostriatal neurons from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity and neuroinflammation. Stereological analyses showed that chronic treatment with WIN (4 mg/kg, i.p.), initiated 24 hr after MPTP administration, protected against MPTP-induced loss of tyrosine hydroxylase positive (TH⁺) neurons in the substantia nigra pars compacta (SNc) independently of CB₁ cannabinoid receptor activation. The neuroprotective effect of WIN was accompanied by increased DA and 3,4-dihydroxyphenylacetic acid (DOPAC) levels in the SNc and dorsal striatum of MPTP-treated mice. Three days post-MPTP, we found significant microglial activation and up-regulation of CB₂ cannabinoid receptors in the ventral midbrain. Treatment with WIN or the CB₂ receptor agonist JWH015 (4 mg/kg, i.p.) reduced MPTP-induced microglial activation, whereas genetic ablation of CB₂ receptors exacerbated MPTP systemic toxicity. Furthermore, chronic WIN reversed MPTP-associated motor deficits, as revealed by the analysis of forepaw step width and percentage of faults using the inverted grid test. In conclusion, our data indicate that agonism at CB₂ cannabinoid receptors protects against MPTP-induced nigrostriatal degeneration by inhibiting microglial activation/infiltration and suggest that CB₂ receptors represent a new therapeutic target to slow the degenerative process occurring in PD.

Corresponding Author: Andrea Giuffrida, Ph.D., University of Texas Health Science Center at San Antonio, Department of Pharmacology, 7703 Floyd Curl Drive - MC 6205, San Antonio, Texas 78229-3900, Phone: (210) 567-8533, Fax: (210) 567-8024, giuffrida@uthscsa.edu.

*Current address: Department of Pharmacology, University of Colorado Denver, Denver, Colorado

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cannabinoid receptors; neurotoxicity; striatum; substantia nigra; neuroinflammation

INTRODUCTION

Parkinson's disease (PD) is characterized by the degeneration of dopaminergic nigrostriatal neurons, which results in disabling motor disturbances, including bradykinesia, postural instability, resting tremor and rigidity (Pakkenberg *et al.*, 1991; Dauer and Przedborski, 2003). Although environmental and genetic factors have been implicated in the pathogenesis of PD, the latter being particularly relevant to familial (*i.e.* early onset) forms, the etiology of idiopathic (*i.e.* late onset) PD remains elusive. Activation of glial cells and the consequent neuroinflammatory response is increasingly recognized as a prominent neuropathological feature of PD (Banati *et al.*, 1998; Vila *et al.*, 2001; Hirsch *et al.*, 2003; Schapira and Olanow, 2004; Block *et al.*, 2007), and reactive astrocytes and microglia have been observed in the parkinsonian midbrain (McGeer *et al.*, 1988; Forno, 1992). Importantly, neuroinflammation affects not only damaged but also healthy neurons, thus leading to a self-perpetuating cycle of neurodegeneration (Hunot and Hirsch, 2003). This scenario can be modeled in mice using the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which reproduces most PD hallmarks, including loss of midbrain dopamine (DA) neurons (Jackson-Lewis *et al.*, 1995; Langston *et al.*, 1999) and microglial activation (Czlonkowska *et al.*, 1996; Kohutnicka *et al.*, 1998; Kurkowska-Jastrzebska *et al.*, 1999; Vila *et al.*, 2001; Wu *et al.*, 2002). DA replacement therapy, via administration of levodopa and/or DA receptor agonists, alleviates PD symptoms initially, but does not slow or halt the underlying neurodegenerative process (Lang and Obeso, 2004). To date, all drugs with proven neuroprotective activity in animal models of PD have failed to show significant efficacy in clinical trials (Block *et al.*, 2007). Thus, there is an urgent need for neuroprotective agents that can be administered during the course of the disease to prevent or, at least, limit PD progression (Ravina *et al.*, 2003; Wu and Frucht, 2005).

The endocannabinoid system is emerging as a promising pharmacological target for the treatment of PD (Meschler *et al.*, 2001; van der Stelt *et al.*, 2005; Centonze *et al.*, 2007; Kreitzer and Malenka, 2007) and levodopa-associated motor complications (Fox *et al.*, 2002; Sieradzan *et al.*, 2001; Morgese *et al.*, 2007). Cannabinoid agents are neuroprotective in many experimental models of pathological conditions, including excitotoxicity (Marsicano *et al.*, 2003), ischemia (Nagayama *et al.*, 1999) and traumatic brain injury (Panikashvili *et al.*, 2001). In addition, cannabinoids are known to modulate inflammatory responses by regulating microglia function via cannabinoid receptor-dependent and -independent mechanisms (Molina-Holgado *et al.*, 2003; Sancho *et al.*, 2003; Walter and Stella, 2004; Ramirez *et al.*, 2005). Given these observations, we investigated whether chronic treatment with the non-selective cannabinoid receptor agonist WIN55,212-2 (WIN) protected mouse nigrostriatal neurons from MPTP-induced neurotoxicity/neuroinflammation while ameliorating PD-associated motor deficits. Since WIN affects the activity of the dopamine transporter (DAT) (Price *et al.*, 2007), which mediates the uptake of the neurotoxic MPTP metabolite 1-methyl-4-phenylpyridinium (MPP⁺) into DA neurons, we initiated WIN administration 24 hr post-MPTP, a time point when MPP⁺ is already cleared from the brain (Irwin *et al.*, 1989; Przedborski *et al.*, 1992).

MATERIALS AND METHODS

Drugs

JTE907 (JTE), JWH015 (JWH) and WIN were purchased from Tocris (Ellisville, MO). AM251 was from Biomol (Plymouth Meeting, PA). Dimethyl sulfoxide (DMSO) and Triton X-100 from Fisher Scientific (Pittsburgh, PA). All other chemicals, unless otherwise stated, were from Sigma (St. Louis, MO).

Animals and Drug Treatments

All experiments were carried out in 8–12 week-old, male C57BL/6N mice (Harlan; Indianapolis, IN), CB₁ receptor knockout mice (CB₁^{−/−}), heterozygous (CB₁^{+/-}) and wild-type (CB₁^{+/+}) littermates (Marsicano *et al.*, 2002a) or CB₂ receptor knockout mice (CB₂^{−/−}) (Jackson Laboratory; Bar Harbor, ME). CB₁^{−/−} mice carrying a germ-line transmissible deletion of the CB₁ receptor and CB₂ receptor knockout mice (strain name: B6.129P2-Cnr2^{tm1Dgen/J}) were backcrossed for five generations into C57BL/6N (Charles River) and genotyped by PCR. Animals were housed at 22±1°C (12 hour light-dark cycle) for one week before experiments with *ad libitum* access to food and water. All experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio.

Animals received four intraperitoneal (i.p.) injections of either saline or MPTP (20 mg/kg, i.p.) at 2 hr intervals, following previously published guidelines (Przedborski *et al.*, 2001). Vehicle (70% DMSO in saline) or cannabinoid drugs (WIN, 1 or 4 mg/kg; AM251, 1 or 4 mg/kg; JWH, 1 or 4 mg/kg; JTE, 4 mg/kg) were administered chronically (i.p.), once a day for 5 days starting 24 hr after the last injection of MPTP.

Immunohistochemistry

Animals were anesthetized with Avertin and intracardially perfused with 4% paraformaldehyde and 5% sucrose in 0.1 M PBS (pH 7.2) for 10 min. Tissue was post-fixed overnight in 4% paraformaldehyde at 4°C and cryoprotected with 30% sucrose for 24 hr. Coronal serial sections (30 µm) covering the rostro-caudal extent of the ventral midbrain or spleen were processed for free-floating immunohistochemistry as described (Ho and Blum, 1998). The following primary antibodies were used: anti-tyrosine hydroxylase (TH; 1:1000, Pel-Freez Biologicals; Rogers, AR), anti-CB₂ (rabbit antiserum prepared against a 14 amino acid peptide from human CB₂; 1:900, Alpha Diagnostics #CB21-A; San Antonio, TX) (Van Sickle *et al.*, 2005), anti-macrophage antigen complex-1 (MAC-1; 1:500, Serotec; Oxford, United Kingdom) or anti-glial fibrillary acidic protein (GFAP; 1:200, Biomedex; Foster City, CA). Incubations with primary antibodies diluted in blocking buffer were carried out for 48 hr at 4°C. Sections were washed in PBS and incubated in biotinylated goat anti-rabbit or anti-rat IgG (Vector Laboratories, Burlingame, CA) for 2 hr at room temperature, followed by ExtrAvidin for 1 hr at room temperature and the color reaction developed with 3,3'-diaminobenzidine tetrachloride (0.25 mg/ml). For fluorescent immunocytochemistry, slide-mounted 20 µm cryosections were incubated in primary antibodies overnight at 4°C, washed in PBS and incubated in fluorescein-conjugated goat anti-rat (for MAC-1) or Texas red goat anti-rabbit (for CB₂ receptors) (Vector Laboratories; Burlingame, CA). Images were captured with an Axioplan 2 Imaging microscope (Carl Zeiss Inc.; Göttingen, Germany), equipped with a Zeiss AxioCam HRc camera and analyzed with Axiovision v.3.0 software (Carl Zeiss Inc., Thornwood, NY). For Nissl staining, adjacent slide-mounted sections (30 µm) were dried overnight at room temperature, stained with thionin for 1 min, dehydrated in alcohol/xylene and coverslipped.

Unbiased Stereology

Stereological analyses of TH positive (TH⁺) neurons in the substantia nigra pars compacta (SNc) were carried out by a blind experimenter using the optical fractionator method (West, 1993), an unbiased method of cell counting that is independent of the volume of the brain area considered and the size of neurons being counted. The SNc was delineated at 10x magnification using published atlases for anatomical landmarks (Franklin and Paxinos, 1997; Hof et al., 2000). TH⁺ neurons were counted 7 days after the last injection of MPTP from every 4th serial section (30 μ m) using an Axioplan 2 Imaging microscope equipped with a DEI-750 CE video camera (Optronics; Goleta, CA), connected to a LEP MAC5000 motorized stage controller (Ludl Electronic Products; Hawthorne, NY) and interfaced to a Dell Dimension 8100 workstation (Dell; Austin, TX, USA) with Stereo Investigator software (Microbrightfield; Williston, VT). A counting frame of x = 100 μ m and y = 100 μ m was incorporated along with steps involving movements of x = 140 μ m and y = 140 μ m.

Western blotting

Dorsal striata and ventral midbrains were homogenized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM Na₃VO₄, 10 mM NaF, 1 mM EDTA, 0.1% Triton X-100, 0.1% SDS) containing protease inhibitors. Equal amounts of protein were separated by SDS-polyacrylamide gel (7.5% or 10% for MAC-1 immunoblotting) and transferred to nitrocellulose membranes (0.45 μ m, Millipore; Billerica, MA). Non-specific binding was blocked with TBS-T (50 mM Tris-HCl pH 7.6, 150 mM NaCl, and 0.1% Tween-20) containing 5% non-fat milk for 1 hr at room temperature. The membranes were then incubated overnight at 4°C with one of the following primary antibodies: anti-TH (1:1000), anti-CB₂ (1:500) (Van Sickle *et al.*, 2005), anti-MAC-1 (1:1000), anti-GFAP (1:500, Dako; Carpinteria, CA) or anti- β -actin (1:3000). After 3 washes in TBS-T, membranes were incubated with goat anti-mouse, anti-rabbit or anti-rat HRP (1:2000, Santa Cruz Biotechnology; Santa Cruz, CA) for 1 hr at room temperature. The signal was visualized using an ECL chemiluminescence kit (Amersham Biosciences/GE Healthcare; Piscataway, NJ), followed by densitometry using NIH Image v.1.63 software (U.S. NIH; <http://rsb.info.nih.gov/ni-image>). Incubations in the presence of CB₂ blocking peptide (10 μ g/ml, Alpha Diagnostics #CB21-P; San Antonio, TX) or in the absence of primary antibodies were used as negative controls.

Neurochemistry

Quantification of DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) was carried out as described (Fernandez *et al.*, 2006). Briefly, frozen dorsal striatum and ventral midbrain tissue samples were homogenized in ice-cold 0.1 M perchloric acid (HClO₄) containing 10 ng/mL of 2,3-dihydroxybenzoic acid (DHBA) as an internal standard and centrifuged at 12,000 rpm for 3 min. An aliquot of the supernatant was filtered through a 0.45 μ m microcentrifuge filter (Millipore; Billerica, MA) and centrifuged at 12,000 rpm for 1 minute. DA and DOPAC were separated using a high-performance liquid chromatography (HPLC) system consisting of a dual piston pump (Solvent Delivery Module-Model 580), a refrigerated autosampler (Model 540) and a Coulochem II dual potentiostat electrochemical detector (Model 500). Data collection and system control were performed using a PC-based data station (Model 500). Separation of DA and DOPAC was achieved on a HR-80 reverse phase C18 column (4.6 $\times$ 80 mm) at a flow rate of 1.0 ml/min. The mobile phase (pH 3.1) consisted of 75 mM sodium phosphate monobasic, 4.0 mM heptanesulfonic acid, 25 μ M EDTA, 0.01% triethylamine and 6% acetonitrile (v/v). Analytes were detected on a dual electrode analytical cell (Model 5011A) with the first electrode (E1) set at -50 mV and the second electrode (E2) set to oxidize catecholamines and its metabolites at +280 mV. A guard cell (Model 5020) was placed between the pump and the autosampler at a potential of

+350 mV to oxidize contaminants in the mobile phase. All equipment was from ESA Biosciences (Chelmsford, MA).

Inverted grid test

Behavioral measures were carried out in 4 groups of mice (saline+vehicle, MPTP+vehicle, saline+WIN, MPTP+WIN) 7 days after injection of MPTP or saline in a sound-isolated room, illuminated with an infrared light. Forepaw step length and percentage of step faults were monitored using an inverted grid as described previously (Tillerson and Miller, 2003; Fleming *et al.*, 2004). Briefly, animals were placed in the center of the grid (total size: 12 cm² with 0.5 cm² mesh openings, at a height of 20 cm above the table-top). The grid was then inverted and the animals were videotaped for 1 min while hanging upside down. Two observers blind to the treatment rated each trial for forepaw step distance (number of grid openings crossed at each step) and percentage of forepaw step faults using a video recorder with frame-by-frame motion as described (Tillerson and Miller, 2003). A step fault was defined as the unsuccessful placement of the forepaw during a weight-shifting movement, thus requiring replacement of the paw on the grid to regain weight-bearing control (Tillerson and Miller, 2003). The percent of forepaw faults was calculated by dividing the number of unsuccessful forepaw steps by the total number of attempted steps.

Data Analysis

All data were analyzed using Prism v.4 (GraphPad Software; San Diego, CA). Differences among means were analyzed by Student's *t* test or one or two-way ANOVA followed by Bonferroni's or Newman-Keuls' post-hoc tests. The threshold for statistical significance was set at $p < 0.05$. All data are expressed as mean $\pm$ SEM.

RESULTS

WIN attenuates MPTP-induced degeneration of nigrostriatal DA neurons

In all experimental groups, TH⁺ neurons in the SNc were counted 7 days after systemic administration of MPTP (4x 20 mg/kg, i.p., 2 hr between injections) using unbiased stereological analysis. As shown in Fig. 1, MPTP caused an ~80% loss of TH⁺ neurons relative to saline-treated animals ($p < 0.001$). Chronic administration of WIN (once a day for 5 days, starting 24 hr after the last MPTP injection) had no effect on MPTP-induced neurotoxicity when given at the dose of 1 mg/kg (i.p.), whereas an ~55% higher TH⁺ cell count ($p < 0.05$, compared to MPTP-treated controls) was observed at the dose of 4 mg/kg (Fig. 1). These data were confirmed by stereological analysis of Nissl-stained neurons, which showed an ~60% cell loss after MPTP (MPTP+vehicle, 5,588 $\pm$ 292.8; saline+vehicle, 13,982 $\pm$ 133.1) and a significant increase (>30%) of Nissl⁺ neurons in MPTP-treated mice following WIN (4 mg/kg, i.p.) administration (MPTP+WIN, 8,508 $\pm$ 198.7; $p < 0.001$, ANOVA followed by Bonferroni's multiple comparisons post-test; $n = 4$ per treatment). WIN did not alter the number of TH⁺ or Nissl stained neurons in saline-treated animals (saline+vehicle, 13,982 $\pm$ 133.1; saline+WIN, 13,277 $\pm$ 523.2). The neuroprotective effect of WIN did not result from TH⁺ up-regulation, as WIN did not alter TH expression in the ventral midbrain of saline-treated mice (data not shown).

MPTP treatment decreased striatal DA and DOPAC content by 66% and 70%, respectively (Fig. 2A). A similar effect was observed in the ventral midbrain, where MPTP reduced DA and DOPAC levels by 69% and 56%, respectively (Fig. 2B). Compared to MPTP-treated mice receiving vehicle, chronic WIN (4 mg/kg, i.p.) did not reverse MPTP-induced DA and DOPAC depletion in the dorsal striatum but produced a 41% increase of DA levels in the ventral midbrain of MPTP-treated mice (Fig. 2). DOPAC/DA ratios were not affected by any of the treatments (Fig. 2).

WIN-induced neuroprotection is CB₁ receptor-independent

We next investigated the pharmacological mechanisms underlying WIN-induced neuroprotection by testing whether the CB₁ receptor antagonist AM251 (1 and 4 mg/kg, i.p.; 15 min before WIN) was able to reverse WIN effect. At 1 mg/kg, AM251 did not alter MPTP-induced DA cell loss (data not shown). However, when given at 4 mg/kg, either alone or in combination with WIN, AM251 produced 100% mortality among MPTP-treated mice. To overcome this problem, we carried out our stereological analyses in CB₁ receptor knockout (CB₁^{-/-}) mice. Although the number of TH⁺ neurons in the SNc did not differ between untreated CB₁^{+/+} and CB₁^{-/-} mice (CB₁^{+/+}, 9356±531; CB₁^{-/-}, 9781±713), CB₁^{-/-} mice were significantly more resistant to MPTP neurotoxicity, as >60% more TH⁺ neurons were observed in these animals 7 days post-MPTP relative to MPTP-treated wild-type littermates (Fig. 3). MPTP-treated CB₁^{+/+} mice had a slightly higher number of TH⁺ neurons in the SNc when compared to CB₁^{+/+} wild-type controls (CB₁^{+/+}, 2,765±181.8; CB₁^{+/+}, 1,955±363.9); this trend, however, did not reach statistical significance. Chronic administration of WIN (4 mg/kg, i.p.) to MPTP-treated CB₁^{-/-} mice produced a significant neuroprotective effect (p<0.001), which was similar in extent to that observed in wild-type controls (Fig. 3), suggesting that WIN protects against MPTP-induced neurotoxicity independently of CB₁ receptor activation.

WIN inhibits MPTP-induced microglia activation via CB₂ receptors

Since the microglial response to MPTP peaks and declines prior to maximal DA cell loss (Liberatore *et al.*, 1999) and pharmacological inhibition of their activation with minocycline protects against MPTP-induced nigrostriatal degeneration (Wu *et al.*, 2002), we next investigated whether WIN was neuroprotective by reducing the microglial response via activation of CB₂ receptors. CB₂ receptor expression was significantly up-regulated 3 days post-MPTP in the ventral midbrain, but not in the dorsal striatum (Fig. 4A). The specificity of this response was determined by: 1) pre-absorbing the CB₂ antibody with a blocking peptide, which abolished the CB₂ signal (data not shown); 2) assessing CB₂ protein expression in the spleen of CB₂^{-/-} mice by Western blot, which was reduced by 97% (p<0.001; unpaired *t*-test) as compared to wild-type controls (in arbitrary units expressed as a ratio of CB₂-to-β-actin: CB₂^{-/-}, 2.0±1.1; CB₂^{+/+}, 58.6±5.0); 3) immunohistochemical staining of spleen from CB₂^{+/+} and CB₂^{-/-} mice (Fig. 4C). Increased CB₂ receptor expression was accompanied by an up-regulation of MAC-1 (p<0.01), a marker of activated microglia, in the same brain region (Fig. 5). Double-label immunofluorescence showed that CB₂ receptors were co-localized with MAC-1 (Fig. 4B). A significant increase of MAC-1 immunoreactivity was also observed in the dorsal striatum of MPTP-treated mice (data not shown).

Treatment with a neuroprotective dose of WIN (4 mg/kg, i.p.) reduced MAC-1 immunostaining (Fig. 5A,B) and expression (Fig. 5C) back to control levels in the SNc at day 3 post-MPTP, whereas it had no significant effect in the dorsal striatum (data not shown). A similar response was obtained after administration of the CB₂ receptor agonist JWH (4 mg/kg, i.p., same regimen as WIN) (Fig. 5C). The CB₂ receptor antagonist JTE (4 mg/kg, i.p.; 20 min before WIN) reversed the WIN-mediated reduction of MAC-1 expression in MPTP-treated mice and increased MAC-1 immunoreactivity when given alone (Fig. 5C). Unfortunately, we could not determine the effects of JWH alone or JTE+WIN on TH⁺ cell counts (measured at day 7 post-MPTP), as all MPTP-treated mice receiving JWH or JTE died within 4–5 days. Also, all MPTP-treated CB₂^{-/-} mice (n=6) died within 24–48 hr indicating that genetic ablation of CB₂ receptors dramatically increases the sensitivity to MPTP-induced systemic toxicity. Thus, we were not able to assess the neuroprotective effects of WIN on TH⁺ neurons in these animals. Nevertheless, WIN decreased MAC-1

levels by 40% in the ventral midbrain of MPTP-treated CB₁^{-/-} mice, indicating that the ability of WIN to reduce microglial activation is CB₁-independent (data not shown).

Since PD is accompanied by an elevation of reactive astrocytes (Forno, 1992), and a similar phenomenon is observed in mice within 3–7 days following MPTP administration (Liberatore *et al.*, 1999), we assessed the effects of WIN on the astrocyte marker GFAP 7 days after MPTP injection. MPTP increased GFAP immunostaining and protein expression in the ventral midbrain; this effect, however, did not reach statistical significance and was unaltered by chronic treatment with WIN (4 mg/kg, i.p.) (data not shown). GFAP up-regulation was also observed in the dorsal striatum of MPTP-treated mice ($p < 0.01$; Student's *t*-test, data not shown). As in the case of the ventral midbrain, this response was not reversed by chronic WIN (data not shown).

WIN reverses MPTP-induced motor deficits

Previous studies have shown that MPTP administration to mice causes motor abnormalities, consisting of shortened forelimb step length and increased step faults, which are correlated with markers of nigrostriatal degeneration and reversed by L-DOPA (Tillerson and Miller, 2003; Fleming *et al.*, 2006). To assess whether the neuroprotective effect of WIN on TH⁺ neurons in MPTP-treated mice was accompanied by functional recovery, we measured forelimb step length and percentage of forepaw step faults in 4 experimental groups (saline + vehicle, MPTP + vehicle, WIN + saline, MPTP + WIN) 7 days post-MPTP using the inverted grid test as previously described (Tillerson *et al.*, 2002; Tillerson and Miller, 2003). As illustrated in Fig. 6, the mean step length score for MPTP-treated mice was significantly lower than in saline-treated controls, and chronic WIN treatment reversed this motor abnormality to control levels (Fig. 6A). Similarly, WIN administration reversed the MPTP-induced increase in percentage of forepaw faults (Fig. 6B). In both tests, WIN had no effect when administered to saline-treated animals.

DISCUSSION

Our data show that chronic administration of the cannabinoid agonist WIN protects mouse nigrostriatal DA neurons from the neurodegenerative effects induced by the neurotoxin MPTP. MPTP-treated mice receiving WIN over 5 days showed ~55% more TH⁺ neurons in the SNc compared to vehicle-injected controls. Despite this robust effect, the number of TH⁺ neurons in the SNc of mice post-treated with WIN was still significantly lower than that of vehicle-treated controls, suggesting that this dose of WIN was not able to rescue all TH⁺ neurons in this area. However, we cannot exclude that a greater protection could have been achieved with a different regimen of WIN (*e.g.* if it had been administered more frequently or for a longer time).

In agreement with previous studies (Centonze *et al.*, 2007), WIN-induced alteration of TH expression cannot account for the neuroprotective effect of this drug, as WIN did not upregulate TH expression in the ventral midbrain of saline-treated mice (data not shown). The effect of WIN was confirmed by Nissl staining, which showed a significant increase of Nissl⁺ neurons in the SNc of WIN-treated animals compared to controls. Furthermore, chronic WIN treatment was accompanied by a significant elevation of DA levels in the ventral midbrain of MPTP-treated mice (41%), whereas we observed only a trend (14% increase) in the striatum. This elevation was independent from drug-induced alterations in DA turnover, as the DOPAC/DA ratios remained unchanged following chronic WIN. Together, these findings suggest that the neuroprotective effect of WIN is not accompanied by a full functional recovery of DA neurons in the ventral midbrain, which appear to synthesize DA at a lower rate. Our data are also in agreement with the study of Garcia-Arencibia *et al.* (2007), which found that systemic administration of a similar dose of WIN

(3 mg/kg) over two weeks did not reverse the DA depletion caused by the neurotoxin 6-OHDA in rat striatum, and suggest that WIN is more effective at: (1) protecting the cell bodies rather than the nerve terminals of nigrostriatal neurons, and (2) slowing down the neurodegenerative process rather than promoting axonal regeneration *ex novo*.

The effects of the CB₁ receptor antagonist AM251 on WIN-induced neuroprotection could not be studied, as all MPTP-treated animals injected with AM251 alone or in combination with WIN died within 48 hr. While the precise cause for this mortality remains unknown, it may be contingent upon side effects exerted by MPTP in addition to its well-known toxicity on DA neurons (Przedborski et al., 2001), such as hypothermia and disruption of cardiac function (Wilson et al., 1991; Moy et al., 1998). These effects may have been further exacerbated by the administration of cannabinoid agents, which, in turn, affect body temperature and cardiovascular function (Hillard, 2000). Interestingly, genetic ablation of CB₁ receptors did not increase MPTP-induced mortality; rather, the higher number of TH⁺ neurons observed in MPTP-treated CB₁^{-/-} mice versus wild-type littermates subjected to the same MPTP regimen suggests that CB₁ knockout mice are more resistant to MPTP-induced nigrostriatal degeneration. This resistance is unlikely to result from altered MPP⁺ uptake since DAT activity is CB₁ receptor-independent (Price et al., 2007) and is unchanged in CB₁^{-/-} mice (Cossu et al., 2001; Houchi et al., 2005). On the other hand, we cannot exclude that the deletion of CB₁ receptors may be accompanied by compensatory mechanisms affecting the activity of monoamine oxidase (MAO) and, consequently, the conversion of MPTP to MPP⁺ and/or that higher availability of endocannabinoids for binding sites other than CB₁ receptors (van der Stelt and Di Marzo, 2004; O'Sullivan, 2007) may account for, or contribute to, MPTP resistance. Nevertheless, we found that chronic administration of WIN to CB₁^{-/-} mice still protected TH⁺ neurons from MPTP-induced insult to the same extent as in wild-type littermates. These results indicate that CB₁ receptors are not involved in WIN-mediated neuroprotection against MPTP neurotoxicity and confirm previous reports in 6-OHDA-treated rats showing that the neuroprotective effects of cannabinoids are CB₁-independent (Lastres-Becker et al., 2005; Garcia-Arencibia et al., 2007). Thus, the view that CB₁ receptors play a neuroprotective role in the brain, as suggested by studies carried out in other models of pathological disorders, including stroke, excitotoxicity and brain trauma (Shen et al., 1996; Nagayama et al., 1999; Panikashvili et al., 2001; Marsicano et al., 2003), cannot be generalized to the MPTP mouse model of PD.

Previous investigations show that MPTP administration in rodents produces a robust glial reaction, mainly characterized by activated microglia and reactive astrocytes in the ventral midbrain. A similar reaction has been reported in the brain of PD patients (Vila et al., 2001). Although the glial response is thought to promote the release of trophic factors, which might be important to support and/or repair damaged brain regions (Liberatore et al., 1999), gliosis has been also associated with deleterious events, including production of pro-inflammatory prostaglandins and cytokines (Banati et al., 1993; Gehrmann et al., 1995), which can significantly contribute to the underlying neurodegenerative process in PD (Herrera et al., 2000; Kim et al., 2000; Wu et al., 2002). As expected in our experimental model, MPTP administration significantly increased GFAP immunostaining and expression in the dorsal striatum and, to a lesser extent, in the ventral midbrain; on the other hand, WIN had no effect on GFAP when given either alone or after MPTP. This lack of effect does not rule out the possibility that WIN might modulate other aspects of astrocytic function (Aguado et al., 2006). Indeed, cannabinoids can inhibit the release of pro-inflammatory mediators from activated astrocytes (Ortega-Gutierrez et al., 2005; Mestre et al., 2006), disrupt the astroglia-mediated amino acid transport (Shivachar, 2007) and reduce MPTP-induced up-regulation of S100B, a calcium/zinc-binding protein promoting astrocyte proliferation (Iuvone et al., 2007). Thus, the ability of cannabinoids to affect MPTP-induced astrocytic responses may differ significantly between in vitro and in vivo studies.

It is worth mentioning that cannabinoid compounds can be neuroprotective independent of cannabinoid receptor activation via their antioxidant properties (Marsicano et al., 2002b; Sagredo et al., 2007) and that antioxidants have neuroprotective effects in animal models of PD (Mandel et al., 2003). However, this is not the case of WIN, which lacks the phenolic moieties conferring the antioxidant activity observed with other cannabinoids (Marsicano et al., 2002b).

Interestingly, administration of the neuroprotective dose of WIN (4 mg/kg) was accompanied by a remarkable suppression of MPTP-induced microglial activation/infiltration in the ventral midbrain 3 days after MPTP, whereas the low, non-protective dose of WIN (1 mg/kg) had no effect. Most likely, the suppressive effect of WIN on microglia was mediated through cannabinoid CB₂ receptors, as it was mimicked by the CB₂ agonist JWH (which is more selective than WIN for CB₂ receptors) and reversed by the CB₂ antagonist JTE; this drug, however, enhanced MAC-1 expression when given alone, making difficult the interpretation of this result from a pharmacological point of view. We also found that (1) CB₂ expression in the ventral midbrain was elevated 3 days post-MPTP, a time point corresponding to heightened microglia reactivity in the CNS (Liberatore et al., 1999), and (2) as previously reported (Benito et al., 2003; Walter et al., 2003), CB₂ receptors were expressed by activated microglia. To our knowledge, this is the first report showing that stimulation of CB₂ receptors significantly reduces microglial infiltration/activation in an *in vivo* model of PD. These findings agree with other studies showing that CB₂ receptor expression increases following neuroinflammatory events (Esposito et al., 2007) and that cannabinoid agonists can modulate microglia mobility via activation of CB₂ receptors *in vitro* (Walter et al., 2003) and *in vivo* (Ramirez et al., 2005). Thus, WIN may exert its neuroprotective actions by reducing the deleterious effects triggered by microglial activation/infiltration, which ultimately contribute to the demise of DA neurons in the SNc (Liberatore et al., 1999; Dehmer et al., 2000; Wu et al., 2002). Unfortunately, we were not able to assess the effects of JWH on TH⁺ neurons at the time of maximal cell loss (i.e. 7 days post-MPTP) or whether the CB₂ antagonist JTE or genetic ablation of CB₂ receptors reversed the beneficial action of WIN, since all animals receiving JWH or JTE died within 4–5 days post-MPTP, and MPTP-treated CB₂^{-/-} mice died within 24–48 hr. Nevertheless, these findings suggest that CB₂ receptors are critical for protecting mice against the systemic toxicity of MPTP. The hypothesis that CB₂ receptor-mediated suppression of microglial activation contributes to WIN neuroprotective effects is supported by other studies showing that cannabinoid agonists block β -amyloid-induced activation of microglia cells and the associated loss of neuronal markers via a CB₂-dependent mechanism (Ramirez et al., 2005). Furthermore, stimulation of CB₂ receptors reduces the neuroinflammatory process occurring in animal models of amyotrophic lateral sclerosis (Bilsland et al., 2006; Kim et al., 2006) and multiple sclerosis (Arevalo-Martin et al., 2003; Eljaschewitsch et al., 2006). Further studies are necessary to investigate the possible role played by other non-CB₁/CB₂ receptor subtypes in WIN-mediated neuroprotection in PD.

Although MPTP causes pathophysiological alterations in mice that are similar to those reported in idiopathic PD, this neurotoxin produces transient hypokinetic behavior and inconsistent motor deficits that disappear within a few days following its administration (Heikkila and Sonsalla, 1992; Fredriksson et al., 1997; Rozas et al., 1998; Spooen et al., 1998; Sedelis et al., 2001; Tillerson et al., 2002). To reveal behavioral correlates to MPTP-induced lesions, we used the grid performance test (Tillerson and Miller, 2003) and measured the average forepaw step distance and percentage of forepaw faults, two behavioral measures sensitive to nigrostriatal damage and DA dysfunction (Tillerson and Miller, 2003; Fleming and Chesselet, 2006). In MPTP-treated mice we observed a significant reduction of the average forepaw step width and a higher percentage of step faults compared to saline-injected controls. Chronic treatment with a neuroprotective dose of

WIN (4 mg/kg, i.p.) reversed MPTP-induced motor deficits, whereas the lower dose of WIN (1 mg/kg, i.p.) had no effect (data not shown). This functional recovery cannot be ascribed to possible hypolocomotive effects of WIN, as all behavioral tests were performed 24 hr after the last injection of WIN (*i.e.* 7 days post-MPTP), thus excluding a direct interference of the drug on locomotor activity via stimulation of CB₁ receptors. Furthermore, each experimental group was equally active, without any differences in the total number of steps being observed (data not shown). Changes in CB₁ receptor sensitivity can also be excluded since saline-treated animals receiving WIN alone did not differ from controls in both behavioral tests.

In summary, our results indicate that the cannabinoid agonist WIN protects against MPTP-induced neurotoxicity and the ensuing functional deficits even when administered once the insult has been initiated. This characteristic is of particular importance, as the lack of PD biomarkers and the difficulties of early diagnosis make the pharmacotherapy of PD possible only when DA neuronal loss is advanced and the first symptoms have appeared. Our data also suggest that CB₂ receptors constitute an important component of PD pathophysiology and that activation of these receptors may have therapeutic applications to prevent the neurodegenerative process occurring in PD.

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Fig. 1. Effect of WIN on MPTP-induced loss of TH⁺ neurons in the SNc. Saline or MPTP (4x 20 mg/kg, i.p.) were administered every 2 hr on day 0 to CB₁^{+/+} mice. Vehicle or WIN (1 mg/kg, i.p. or 4 mg/kg, i.p.) were administered every day over a 5 day period, starting 24 hr after the last injection of saline or MPTP. Animals were sacrificed on day 7. (A) Representative microimages of midbrain immunostained for TH (scale bar = 100 μm). (B) Unbiased stereological quantification of TH⁺ neurons in the SNc. ###p<0.001 compared to saline-treated control; *p<0.05 as shown (ANOVA followed by Bonferroni's multiple comparisons post-test; n=5 per treatment).

Fig. 2.

Measurement of DA and DOPAC levels and DA turnover (DOPAC/DA) in the dorsal striatum (A) and ventral midbrain (B). Saline or MPTP (4x 20 mg/kg, i.p.) were administered every 2 hr on day 0. Vehicle or WIN (4 mg/kg, i.p.) were administered every day over a 5 day period, starting 24 hr after the last injection of MPTP. Animals were sacrificed on day 7 post-MPTP treatment. * $p<0.05$ and ** $p<0.01$ compared to saline +vehicle; # $p<0.05$ compared to MPTP+vehicle (ANOVA followed by Newman-Keuls' multiple comparisons post-test; $n=4$ per treatment).

Fig. 3.

Effect of genetic ablation of CB₁ on WIN-mediated protection against MPTP-induced loss of TH⁺ neurons. CB₁^{+/+} or CB₁^{-/-} mice received 4 injections of MPTP (20 mg/kg, i.p.) every 2 hr on day 0. Vehicle or WIN (4 mg/kg, i.p.) were administered every day over a 5 day period, starting 24 hr after the last injection of MPTP. Animals were sacrificed on day 7 and TH⁺ neurons were quantified via unbiased stereology. ###p<0.001 compared to saline-treated CB₁^{+/+} (dashed line); *p<0.05 and ***p<0.001 as shown (ANOVA followed by Bonferroni's multiple-comparisons post-test; n=5 per treatment).

Fig. 4.

Effects of MPTP on brain CB_2 receptor expression. Saline or MPTP (4x 20 mg/kg, i.p.) were administered every 2 hr on day 0 and animals were sacrificed on day 3. (A) Western blot analysis of CB_2 receptor expression in ventral midbrain and dorsal striatum. Bands were quantified by densitometry. ** $p < 0.01$ compared to saline-treated control (unpaired Student's *t*-test; $n = 4$ per treatment). (B) Double-label immunofluorescence confirms that CB_2 receptors (green) and MAC-1 (red) co-localize in the SNc (scale bars = 10 μ m). (C) Immunofluorescence confirms that CB_2 receptors are present in spleen from $CB_2^{+/+}$ but not $CB_2^{-/-}$ mice (scale bars = 10 μ m).

Fig. 5.

Effects of MPTP and WIN on microglial activation/infiltration. Saline or MPTP (4x 20 mg/kg, i.p.) were administered every 2 hr on day 0 and animals were sacrificed on day 3. Vehicle or cannabinoids were administered on days 1–3. (A) Representative microimages of the ventral midbrain immunostained for MAC-1 (scale bar = 100 μ m). (B) Higher magnification image of the SNc immunostained for MAC-1 3 days post-MPTP treatment (scale bar = 25 μ m). (C) Densitometry of MAC-1 expression in ventral midbrain following western blotting. * p <0.05 and ** p <0.01 compared to saline-treated control (dashed line); *** p <0.001 compared to MPTP+vehicle (ANOVA followed by Bonferroni's multiple comparisons post-test; n =3 per treatment).

Fig. 6. WIN reverses MPTP-induced motor deficits. Forepaw step length (A) and percentage of forepaw step faults (B) were measured in mice receiving saline or MPTP and post-treated with vehicle or WIN (4 mg/kg, i.p.) as previously described. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as shown (ANOVA followed by Newman-Keuls' post-test; $n = 8$ per treatment).