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**Supplementary Material**

5.DC1

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Cannabinoid Receptor Type 1 Protects Nigrostriatal Dopaminergic Neurons against MPTP Neurotoxicity by Inhibiting Microglial Activation

Young C. Chung,*‡,† Eugene Bok,*‡,2 Sue H. Huh,*†,§ Ju-Young Park,§ Sung-Hwa Yoon,* Sang R. Kim,*† Yoon-Seong Kim,‖ Sungho Maeng,‡ Sung Hyun Park,§ and Byung K. Jin*

This study examined whether the cannabinoid receptor type 1 (CB1) receptor contributes to the survival of nigrostriatal dopaminergic (DA) neurons in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson’s disease. MPTP induced significant loss of nigrostriatal DA neurons and microglial activation in the substantia nigra (SN), visualized with tyrosine hydroxylase or macrophage Ag complex-1 immunohistochemistry. Real-time PCR, ELISA, Western blotting, and immunohistochemistry disclosed upregulation of proinflammatory cytokines, activation of microglial NADPH oxidase, and subsequent reactive oxygen species production and oxidative damage of DNA and proteins in MPTP-treated SN, resulting in degeneration of DA neurons. Conversely, treatment with nonselective cannabinoid receptor agonists (WIN55,212-2 and HU210) led to increased survival of DA neurons in the SN, their fibers and dopamine levels in the striatum, and improved motor function. This neuroprotection by cannabinoids was accompanied by suppression of NADPH oxidase reactive oxygen species production and reduced expression of proinflammatory cytokines from activated microglia. Interestingly, cannabinoids protected DA neurons against 1-methyl-4-phenyl-pyridinium neurotoxicity in cocultures of mesencephalic neurons and microglia, but not in neuron-enriched mesencephalic cultures devoid of microglia. The observed neuroprotection and inhibition of microglial activation were reversed upon treatment with CB1 receptor selective antagonists AM251 and/or SR14,716A, confirming the involvement of the CB1 receptor. The present in vivo and in vitro findings clearly indicate that the CB1 receptor possesses anti-inflammatory properties and inhibits microglia-mediated oxidative stress. Our results collectively suggest that the cannabinoid system is beneficial for the treatment of Parkinson’s disease and other disorders associated with neuroinflammation and microglia-derived oxidative damage. The Journal of Immunology, 2011, 187: 6508–6517.

Parkinson’s disease (PD) is a common neurodegenerative disorder characterized by the progressive degeneration of nigrostriatal dopaminergic (DA) neurons. The most prominent biochemical changes in PD involve reduction of the striatal dopamine levels that may result in abnormal motor behavior, including resting tremor, rigidity, and bradykinesia (1, 2). Although the specific cause of PD is yet to be established, a growing body of evidence supports the theory that microglial activation-derived oxidative stress increases the risk of PD (3). In the substantia nigra (SN) of PD patients and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models of PD, key enzymes involved in reactive oxygen species (ROS) production, such as microglial NADPH oxidase, are upregulated in damaged areas and contribute to DA neuronal death (4–7). In addition, proinflammatory cytokines, such as IL-1β and TNF-α, are augmented in PD patients (8, 9) and participate in DA neuronal death in the MPTP model of PD (10, 11).

The cannabinoid receptor type 1 (CB1) receptor is a member of the seven-transmembrane domain G-protein–coupled receptor family (12, 13). CB1 receptor is primarily expressed in glutamatergic neurons and axon terminals of GABA interneurons in CNS (14). CB1 receptor is also found in DA neurons (15) and...
nonneuronal cells, including microglia (16), astrocytes (17, 18), and oligodendrocytes (19). This receptor is activated in response to endogenous ligands, which are mainly derivatives of polyunsaturated fatty acids, including anandamide (arachidonylethanolamide [AEA]) (20, 21) and 2-arachidonoylglycerol (21, 22). Several synthetic cannabinoids, such as HU210 (23, 24) and WIN55,212-2 (24, 25), have been confirmed as ligands of the CB1 receptor. However, the effects of endogenous and synthetic cannabinoids differ (26).

Autoradiography, in situ hybridization (27), and immunohistochemical studies (28) revealed widespread distribution of the CB1 receptor in the mouse brain, including basal ganglia, signifying an important role in the CNS. Cannabinoids and the CB1 receptor exert neuroprotective effects in response to neurotoxic stimuli. Arvanil, a synthetic AEA analog, acts against ouabain-induced excitotoxicity by CB1 receptor activation and prevents neuronal cell death in vivo (29). HU210 displays neuroprotective activity against excitotoxicity in a multiple sclerosis model (30). WIN55,212-2 protects against ventral tegmental area neuronal death under ischemic conditions in vivo and in vitro (31). The actions of these two compounds are inhibited by CB1 receptor antagonists, such as SR141716A, indicative of CB1 receptor-mediated neuroprotection. Interestingly, acute injection of rimonabant (SR14716A) has been shown to rescue DA neurons and improve motor deficits in a 6-hydroxydopamine (6-OHDA) model of PD (32, 33), supporting CB1 receptor-mediated neurotoxicity.

Notably, AEA is increased in the cerebrospinal fluid of PD patients (34) and animal models of PD produced by administration of 6-OHDA (35). In an MPTP-treated macaque model, increases in AEA and 2-arachidonoylglycerol compensate for dopamine depletion (36). Plant-derived cannabinoids, such as δ-9-tetrahydrocannabinol and cannabidiol, protect nigrostriatal DA neurons from 6-OHDA neurotoxicity in vivo in a CB1 receptor-independent manner (37). In the current study, we examined whether activation of the CB1 receptor contributes to neuroprotection of nigrostriatal DA neurons against microglial activation-derived oxidative stress in the MPTP mouse model of PD.

**Materials and Methods**

**Material**

Materials were purchased from the following companies: AM251, HU210, and WIN55,212-2 (Tocris). SR141716A was prepared by following the previously reported method (38). HU210, WIN55,212-2, AM251, and SR141716A were dissolved in DMSO and then diluted with sterile PBS (1:10 solution of DMSO/PBS). The final concentration of all vehicles for treatment on animals was 0.1% DMSO, and there was no neurotoxicity, compared with vehicle-untreated controls.

**Animals and drugs treatment**

All experiments were performed in accordance with the approved animal protocols and guidelines established by Kyung Hee University [KHUASP (SE)-10-030]. Eight-week-old male C57BL/6 mice (23–25 g; Charles River Breeding Laboratory) were used. For MPTP intoxication, mice received four i.p. injections of MPTP (20 mg/kg, free base; Sigma-Aldrich) dissolved in saline at 2-h intervals. The nonselective cannabinoid receptor agonists WIN55212-2 and HU210 received various doses of the drug (0.1, 20 μg/kg body weight once a day, 1 μg/kg body weight once a day, 10 μg/kg once a day, 20 μg/kg twice a day, and 50 μg/kg/once a day) into the peritoneum for 2 d before the MPTP injections and the specified time periods comprising 12 h after the last MPTP injection for 8 d (Supplemental Fig. 1). The CB1 receptor antagonists AM251 and SR141716A received the drug (20 μg/kg once a day) into the peritoneum for 30 min before the CB1 receptor agonist injections. Control mice were injected with CB1 receptor agonists and antagonists alone or the vehicle. WIN55212-2, HU210, AM251, and SR141716A were prepared by following the previously reported method (39).

**Tissue preparation and Immunostaining**

Animals were transcardially perfused with saline solution containing 0.5% sodium nitrate and heparin (10 U/ml) and fixed with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB). Brains were dissected from the skull, postfixed overnight in buffer 4% paraformaldehyde at 4°C, stored in 30% sucrose solution until they sank, and freeze-sectioned with a sliding microtome into 30-μm-thick coronal sections. All sections were collected in six separate series and processed for immunostaining, as described previously (4, 5). In brief, brain sections were rinsed in PBS and incubated overnight at room temperature (RT) with primary Abs, specifically anti–macrophage Ag complex-1 (MAC-1; 1:200 dilution; Serotec, Oxford, U.K.) for microglia and anti-tyrosine hydroxylase (TH; 1:2000 dilution; Pel-Freeze Biologiscals, Rogers, AR) for DA neurons. The following day, sections were rinsed with PBS and 0.5% BSA, incubated with the appropriate biotinylated secondary Ab, and processed with an avidin-biotin complex kit (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Bound antiserum was visualized by treatment with 0.05% diaminobenzidine-HCl and 0.003% hydrogen peroxide in 0.1 M PB. The diaminobenzidine reaction was terminated by rinsing tissues in 0.1 M PB. Labeled tissue sections were mounted on gelatin-coated slides and analyzed under a bright-field microscope (Nikon, Melville, NY). For double-immunofluorescence staining, the sections were incubated in a combination of goat polyclonal Ab to NADPH oxidase subunits mouse anti-p67phox (1:500 dilution; BD Biosciences, San Diego, CA), rabbit anti-p47phox (1:200 dilution; Santa Cruz Biotechnology), or mouse anti-gp91phox (1:500; BD Biosciences), and a rat mAb against MAC-1 (Serotech; 1:200 dilution) overnight at 4°C. After washing in PBS, sections were treated simultaneously with a mixture of FITC-conjugated rabbit anti-rat IgG (1:200 dilution; Vector Laboratories) and Texas Red-conjugated donkey anti- goat IgG (1:200 dilution; Molecular Probes) for 1 h at RT. Slides were mounted with Vectashield medium (Vector Laboratories) and viewed using an IX71 confocal laser scanning microscope (Olympus Optical, Tokyo, Japan). To determine the localization of different Abs in double-stained samples, images were obtained from the same area and merged using interactive software.

**Stereological cell counts**

The total number of TH-positive neurons was counted in the various animal groups at 7 d postinjection (MPTP or saline) using the optical fractionator method performed on an Olympus Computer Assisted Stereological Toolbox system version 2.14 (Olympus Denmark, Ballerup, Denmark) as previously described (4, 5). Actual counting was performed using a 100× oil objective. The total number of neurons was estimated according to the optical fractionator equation (40). More than 300 points over all sections of each specimen were analyzed.

**Densitometric analysis**

As previously described (4, 5), the OD of TH-positive fiber in striatum (STR) was examined at ×5 original magnification using the IMAGE PRO PLUS system (Version 4.0; Media Cybernetics, Silver Spring, MD) on a computer attached to a light microscope (Zeiss Axioskop, Oberkochen, Germany) interfaced with a CCD video camera (Kodak Mega Plus model 1.4 I; Kodak, New York, NY). To control variations in background illumination, the average of background density readings from the corpus callosum was subtracted from that of density readings of the STR for each section. For each animal, the average of all sections was calculated separately before data were statistically processed.

**Measurement of dopamine levels in the STR**

The measurement of dopamine was performed as described previously (4, 5). The samples were analyzed for dopamine, separated with a Waters Symmetry C18 column (5 μm; 150 × 4.6 mm; Waters) at 35°C, and detected electrochemically using a Waters 2465 detector (Waters). The mobile phase consisted of 75 mM NaH2PO4, 1.7 mM 1-octanesulfonic acid, and a rat mAb against MAC-1 (Serotech; 1:200 dilution) overnight at 4°C. After washing in PBS, sections were treated simultaneously with a mixture of FITC-conjugated rabbit anti-rat IgG (1:200 dilution; Vector Laboratories) and Texas Red-conjugated donkey anti-goat IgG (1:200 dilution; Molecular Probes) for 1 h at RT. Slides were mounted with Vectashield medium (Vector Laboratories) and viewed using an IX71 confocal laser scanning microscope (Olympus Optical, Tokyo, Japan). To determine the localization of different Abs in double-stained samples, images were obtained from the same area and merged using interactive software.

**Measurement of MPTP and 1-methyl-4-phenyl-pyridinium levels in the STR**

As described previously (5), striatal MPTP and 1-methyl-4-phenylpyridinium (MPP+) levels were measured by liquid chromatography.
electrospray ionization mass spectrometry. Dissected striatal tissues were sonicated and centrifuged at 9000 rpm for 20 min in chilled 0.1 M perchloric acid (400 μl), and 100 μl supernatant was isocratically eluted through a 150 mm × 1.5 mm internal diameter, 4 μm Zorbax Eclipse XDB-C18 column (Agilent Technologies, Palo Alto, CA) maintained at 23°C at a flow rate of 0.2 ml min⁻¹ for the separation of MPTP and MPP⁺.

Real-time PCR

Animals treated with or without nonselective agonists and/or CB₁ receptor antagonist were decapitated 1 d after injection of MPTP, and the bilateral SN regions were immediately isolated. Total RNA was prepared with RNAzol B (Tel-Test, Friendwood, TX), and reverse transcription was carried out using SuperScript II reverse transcriptase (Life Technologies, Rockville, MD) according to the manufacturer’s instructions. The primer sequences used in this study were as follows: 5’-TGATGTTCCATTA-GACGC-3’ (forward) and 5’-GAGGTCGTGATGTCAGT-3′ (reverse) for IL-1β; 5’-GGACCTGGAGAATCTGAGAG-3’ (forward) and 5’-TGAAGGGAGGCCCATTGGAGA-3′ (reverse) for TNF-α; and 5’-TCCCTCAAGATGTCAGCA-3’ (forward) and 5’-AGA TCCGACAGGATACATT-3′ (reverse) for GAPDH. Real-time PCR reactions were performed in a reaction volume of 20 μl including 1 μl reverse transcription product as a template, 10 μl SYBR Green PCR master mix (Applied Biosystems, Warrington, U.K.), and 20 pmol each primer described above. The PCR amplifications were performed with 40 cycles of 95°C for 30 s and 60°C for 60 s using an ABI 7500 (Applied Biosystems).

Measurement of proinflammatory cytokines

At 3 d from final MPTP treatment, mice treated with or without nonselective cannabinoid receptor agonists and/or CB₁ receptor antagonists were sacrificed, and then SN tissues were isolated. The amount of IL-1β and TNF-α from SN was measured with sandwich ELISA techniques. Tissues were prepared for ELISA as described (4, 5). Equal amounts of protein (100 μg) from each sample were placed in ELISA kit strips coated with the appropriate Ab. Sandwich ELISA was then performed according to the manufacturer’s instructions (BioSource International, Camarillo, CA). The detection limits of IL-1β and TNF-α were 5 pg/ml and 25 pg/ml, respectively.

Neuron-enriched mesencephalic cultures and drug treatment

SD rat ventral mesencephalon tissues were isolated from embryonic day 14 fetal brain and dissected (5). Tissues were cut into small fragments and incubated in Ca²⁺-, Mg²⁺-free HBSS for 10 min at 37°C. Cultures were prepared with 0.1% trypsin solution in Ca²⁺-, Mg²⁺-free HBSS, incubated for an additional 9 min, rinsed twice in DMEM (Life Technologies), supplemented with 10% FBS, 6 mg/ml glucose, 204 μg/ml t-glutamine, and 100 U/ml penicillin/streptomycin, and mechanically triturated. Dissociated cells were plated on 12-mm round aclar plastic coverslips (1.0 × 10⁶ cells/cover slip) precoated with 0.1 mg/ml poly-D-lysine and 4 μg/ml laminin and housed in 24-well culture plates. Cells were maintained in a humidified incubator at 37°C, 5% CO₂ for 24 h. The media of 2-d-old in vitro cultures (days in vitro [DIV] 2) incubated in the absence of serum were replaced with chemically defined serum-free medium composed of Ham’s nutrient mixture (F12-DMEM) and supplemented with 1% insulin, transferrin, selenium, t-glutamine, and penicillin/streptomycin. At 4 DIV, cultures were transferred to defined serum-free medium without insulin, transferrin, selenium, treated MPP⁺ for 48 h, and processed for further experiments.

Cocultures of mesencephalic neurons and microglia

Mesencephalic microglia cultures were prepared from the ventral mesencephalon of embryonic day 14 SD rat brain as previously described (5). Tissues were triturated and plated in 75-cm² T-flasks precoated with poly-ν-lactide at a density of 1 × 10⁶ cells/flask and then maintained in DMEM supplemented with 10% FBS. After 2 to 3 wk, microglia were delaminated from the flasks applied to a nylon mesh to remove astrocytes. At 3 DIV, neuron-enriched mesencephalic cultures plated on 12-mm round aclar plastic coverslips (1.0 × 10⁶ cells/cover slip) precoated with 0.1 mg/ml poly-D-lysine and 4 μg/ml laminin in 24-well culture plates. Cells were maintained in a humidified incubator at 37°C, 5% CO₂ for 24 h. The media of 2-d-old in vitro cultures (days in vitro [DIV] 2) incubated in the absence of serum were replaced with chemically defined serum-free medium composed of Ham’s nutrient mixture (F12-DMEM) and supplemented with 1% insulin, transferrin, selenium, t-glutamine, and penicillin/streptomycin. At 4 DIV, cultures were transferred to defined serum-free medium without insulin, transferrin, selenium, treated MPP⁺ for 48 h, and processed for further experiments.

Statistical analysis

All values are expressed as mean ± SEM. Statistical significance was assessed by two-way ANOVA using Instat 3.05 (GraphPad), followed by Student–Newman–Keuls analyses.
**Results**

**WIN55,212-2 and HU210 protect nigrostriatal DA neurons from the MPTP neurotoxicity in vivo**

After 7 d from last MPTP injection, the mice were sacrificed for immunohistochemical assessments. The brain tissues were immunostained with a TH Ab for detecting DA neurons (Figs. 1A–L, 2A–F). TH immunostaining revealed that four injections of MPTP led to damage in SN (Fig. 1C, 1D) and STR (Fig. 2B) compared with saline-treated control groups (Figs. 1A, 1B, 2A).

DA neuronal cells were quantified as the stereological count of TH-positive cells in the SN and OD of TH-positive fibers in the STR. The quantitative data confirmed that MPTP induces 71% (p < 0.001) DA neuronal death in SN and 77% loss in TH-positive fibers in STR (p < 0.001), respectively, compared with PBS-treated mice as controls. By contrast, mice treated with nonselective cannabinoid receptor agonists WIN55,212-2 and HU210 led to a dramatic increase of TH-positive neurons in SN (Fig. 1E–H) and fibers in STR (Fig. 2C, 2D). Treatment of 0.1 μg/kg and 1 μg/kg WIN55,212-2 and HU210 had no effects (Supplemental Table I), whereas 10 μg/kg WIN55,212-2 and HU210 produced 59% (p < 0.001) and 54% (p < 0.001) increase in TH-positive cells numbers in SN, respectively, compared with MPTP-treated mice (Fig. 1M). Moreover, WIN55,212-2 and HU210 led to 66% (p < 0.001) and 56% (p < 0.001) increase in TH-positive nerve terminals in STR (Fig. 2G).

To ascertain whether this neuroprotective action was mediated by activation of CB1 receptor in vivo, we examined the effect of CB1 receptor antagonists AM251 and SR14716A in MPTP mice. AM251 (20 μg/kg) decreased TH-positive cells numbers in the SN by 47% (p < 0.001) and 55% (p < 0.001) (Fig. 1M) and TH-positive fibers in the STR by 47% (p < 0.001) and 55% (p < 0.001) (Fig. 2G), respectively, compared with MPTP plus
WIN55,212-2-treated or MPTP plus HU210-treated mice. Additional experiments were performed to corroborate CB1 receptor-mediated neuroprotection by using another CB1 receptor antagonist, SR14716A. Consistent with AM251, pretreatment of 20 μg/kg SR14716A attenuated the survival of TH-positive neurons in the SN treated with MPTP plus WIN55,212-2 (36%; p < 0.001) or MPTP plus HU210 (49%; p < 0.001) (Fig. 1M). SR14716A also decreased the protective effect on TH-positive fiber in STR treated with MPTP plus WIN55,212-2 (32%; p < 0.001) or MPTP plus HU210 (44%; p < 0.001) (Fig. 2G), further indicative of CB1 receptor-mediated neuroprotection. As controls, AM251 or SR14716A alone had no effects (Figs. 1M, 2G).

WIN55,212-2 and HU210 increase striatal dopamine levels and improves motor behavior in MPTP mice

We next determined whether WIN55,212-2 and HU210 recover MPTP-induced motor deficits by testing rotarod performance (4). MPTP treatment decreased sustained rotarod time to 11.65 ± 0.53 min compared with that of PBS treatment (Fig. 2H; p < 0.001). By contrast, WIN55,212-2 and HU210 partially improve behavioral dysfunction, which increased the latency to falling to 18.52 ± 0.29 min (Fig. 2H; p < 0.001) and 18.26 ± 0.41 min (Fig. 2H; p < 0.001), respectively.

After rotarod performance test, the mice were sacrificed for measuring striatal dopamine levels using HPLC analysis. In parallel with motor deficits, quantification of striatal dopamine levels revealed that MPTP induces 79% reduction of dopamine levels (Fig. 2H; p < 0.001). By contrast, WIN55,212-2 and HU210 increased striatal dopamine levels in MPTP mice by 48% (Fig. 2H; p < 0.001) and 44% (Fig. 2H, p < 0.001). These behavioral and biochemical effects of WIN55,212-2 and HU210 were significantly reversed by AM251 (Fig. 2H). As controls, WIN55,212-2, HU210, and AM251 alone had no effects on behavior and dopamine levels.

WIN55,212-2 and HU210 do not alter the metabolism of MPTP to MPP+

MPTP neurotoxicity correlates linearly to the striatal levels of MPP+, an active toxic metabolite of MPTP converted by monoamine oxidase-B, in mouse brain (41). To determine whether the observed neuroprotection of WIN55,212-2 and HU210 was attributable to alteration of MPTP conversion to MPP+, we measured the striatal content of MPTP and MPP+ at 2 h after the last injection of MPTP in the presence or absence of WIN55,212-2 and HU210 (Table 1). The reason we chose 2 h after the last injection of MPTP is because striatal levels of MPP+ were peaked at 2 h after the last MPTP injection, and MPTP and MPP+ levels were measured by liquid chromatography electrospray ionization mass spectrometry. Note that there are no changes of striatal MPP+ levels in the absence and presence of CB agonists.

Table 1. The effects of nonselective cannabinoid receptor agonists on MPTP and MPP+ levels (μg/mg protein) in the striata of C57 BL/6 mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MPTP</th>
<th>MPTP + WIN55,212-2</th>
<th>MPTP + HU210</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPTP</td>
<td>0</td>
<td>2.5  ± 0.9</td>
<td>2.6  ± 1.1</td>
<td>2.7  ± 1.2</td>
</tr>
<tr>
<td>MPP+</td>
<td>0</td>
<td>3.4  ± 0.2</td>
<td>3.5  ± 0.3</td>
<td>3.6  ± 0.6</td>
</tr>
</tbody>
</table>

For nonselective cannabinoid receptor agonist treatment, mice received a single injection/day of WIN55,212-2 and HU210 (10 μg/kg body weight) into the peritoneum for 3 d, beginning at 30 min before first MPTP injection. After pretreatment, mice received four i.p. injections of MPTP (20 mg/kg body weight) at 2-h intervals. Striatal tissues were removed at 2 h after the last MPTP injection, and MPTP and MPP+ levels were measured by liquid chromatography electrospray ionization mass spectrometry. Note that there are no changes of striatal MPP+ levels in the absence and presence of CB agonists.

![FIGURE 3. Activation of CB1 receptor inhibits microglial activation and oxidants production in the SN in vivo. Animals receiving vehicle as a control (A–C), or MPTP (D–F), MPTP and WIN55,212-2 (G–I), MPTP and HU210 (J–L), MPTP, WIN55,212-2, and AM251 (M–O), or MPTP, HU210, and AM251 (P–R) were sacrificed 3 d after last MPTP injection. SN tissues were prepared for MAC-1 (A, D, G, J, M, P) and ED-1 (B, E, H, K, N, Q) immunostaining to detect microglia or for hydroethidine (HE) histochemistry to detect the oxidants production (C, F, I, L, O, R). Five to seven animals were used for each experimental group. Insets show higher magnifications of A, B, D, E, G, H, J, K, M, N, P, Q, 200 μm; C, F, I, L, O, R, 150 μm. C, control; M, MPTP; MH, MPTP and HU210; MHA, MPTP, HU210, and AM251; MW, MPTP and WIN55,212-2; MWA, MPTP, WIN55,212-2, and AM251.](http://www.jimmunol.org/previewimage.png)
receptor agonists and antagonists alone had no effect on microglial activation (data not shown).

In MPTP-treated mice, activated microglia produce O$_2^-$ and O$_3^-$-derived oxidants that contributed to DA neuronal death in the SN via oxidative stress (4, 7). Accordingly, we examined whether WIN55,212-2 and HU210 rescued nigral DA neurons by inhibiting MPTP-induced oxidant production. MPTP-treated mice showed a significant increase in the fluorescent products of oxidized hydroethidine (i.e., ethidium accumulation) in the SN at 3 d from MPTP injection (Fig. 3F) compared with PBS-treated mice (Fig. 3C). Treatment with WIN55,212-2 (Fig. 3F) and HU210 (Fig. 3L) diminished ethidium accumulation in MPTP mice. In contrast, AM251 significantly increased the reduced oxidants level in the SN treated with MPTP plus WIN55,212-2 (Fig. 3O) and MPTP plus HU210 (Fig. 3R).

**WIN55,212-2 inhibits MPTP-induced activation of microglial NADPH oxidase in the SN**

NADPH oxidase is composed of cytosolic component, including p47$^{\text{phox}}$, p67$^{\text{phox}}$, Rac-1, and a membrane component, such as gp91$^{\text{phox}}$ (43, 44). This enzyme produces O$_2^-$ and O$_3^-$-derived oxidants through translocation of its subunits from the cytosol to plasma membrane in activated glial cells, which eventually contributes to DA neuronal death in MPTP-treated mice (4). To examine whether WIN55,212-2 affects the activity of NADPH oxidase in the SN, we accomplished the Western blot after separating into membrane and cytosolic components. At 3 d after the final MPTP injection, levels of cytosolic NADPH oxidase subunits (p47$^{\text{phox}}$ and Rac-1) were significantly increased in the membrane fraction (Fig. 4A, 4B; p < 0.01), indicative of translocation and activation of the complex. MPTP-induced translocation of p47$^{\text{phox}}$ (Fig. 4A, 4B; p < 0.01) and Rac-1 (Fig. 4A, 4B; p < 0.01) were dramatically decreased in SN treated with WIN55,212-2. This effect was reversed by AM251, a CB$_2$ receptor antagonist. WIN55,212-2 alone had no effects. Additional double-immunofluorescence staining demonstrated in vivo that, in MPTP-treated SN, the p47$^{\text{phox}}$-, p67$^{\text{phox}}$-, and gp91$^{\text{phox}}$-positive cells (Fig. 4C–E, red) were localized within MAC-1-positive microglia (Fig. 4C–E, green), but not astrocytes or neurons (data not shown).

**WIN55,212-2 and HU210 attenuate MPTP-induced oxidative damages**

ROS derived from NADPH oxidase mediates MPTP neurotoxicity through oxidative damage on cellular components such as nucleic acids, proteins, and lipids (3). The levels of 8-hydroxy-2-deoxyguanosine (8-OHGdG), a marker of oxidative nucleic acid damage, are increased in cerebrospinal fluid of PD patients (45) and MPTP mice (4, 5). To determine the effects of WIN55,212-2 and HU210 on MPTP-induced oxidative damage in nucleic acids, we immunostained with 8-OHGdG. Substantial increases in 8-OHGdG content were evident in the SN 3 d after the final MPTP injection (Fig. 5C, 5D) compared with the SN of PBS-treated controls (Fig. 5A, 5B). This MPTP-induced increase in 8-OHGdG levels was significantly abrogated in the SN treated with WIN55,212-2 (Fig. 5E, 5F) and HU210 (Fig. 5G, 5H). This effect was reversed by AM251 (Fig. 5I–L). As controls, WIN55,212-2, HU210, and AM251 alone had no effects (data not shown).

Oxidative damage to proteins is significantly increased in the SN of PD patients (46, 47) and MPTP mice (5). MPTP-induced protein oxidative damage was assessed by measuring protein carbonylation in the SN. Western blot analysis revealed that the levels of protein carbonyls were significantly increased in MPTP-treated SN compared with controls (Fig. 5M, 5N; p < 0.01). By contrast, treatment with HU210 reduced MPTP-induced increase in the levels of protein carbonyls (Fig. 5M, 5N; p < 0.01). This effect was reversed by AM251 (Fig. 5M, 5N; p < 0.05).

**WIN55,212-2 and HU210 inhibit MPTP-induced expression of IL-1β and TNF-α**

Several lines of evidence have demonstrated that mice expressing a dominant-negative inhibitor of IL-1β-converting enzyme or those deficient in TNF-α are resistant to MPTP-induced neurotoxicity (10, 11). Thus, we examined whether WIN55,212-2 and HU210 affect MPTP-induced expression of IL-1β and TNF-α, resulting in DA neuronal survival. Real-time PCR analysis showed that WIN55,212-2 and HU210 dramatically inhibited MPTP-induced mRNA expression of IL-1β and TNF-α at 1 d after the last MPTP injection (Fig. 6A). As expected, these inhibitory effects were almost completely reversed by AM251.

To confirm that these changes at the mRNA level are reflected in changes at the protein level, we analyzed tissue lysates by ELISA 3 d after the final MPTP injection (Fig. 6B). Similar to real-time PCR results, ELISA analyses showed that the levels of IL-1β and TNF-α protein were significantly increased in the SN of MPTP-treated mice compared with the SN of PBS-treated mice (Fig. 6B). Treatment with CB$_2$ receptor agonists inhibited these MPTP-induced increases, reducing expression of IL-1β by 64 and 55% (p < 0.01; Fig. 6B) and TNF-α by 51 and 38% (p < 0.01; Fig.
protein oxidation reveals that CB1 receptor reduces the protein carbonyls.

FIGURE 5. Activation of CB1 receptor prevents oxidative damages on nucleic acids and proteins in MPTP-treated SN in vivo. At 3 d after the final MPTP injection, 8-OHdG immunostaining (brown) and then performed Nissl staining (blue) in mouse SN treated with PBS (A, B), MPTP (C, D), or MPTP and WIN55,212-2 (E, F), MPTP and HU-210 (G, H), MPTP; WIN55,212-2, and AM251 (I, J), or MPTP; HU210, and AM251 (K, L). B, D, F, H, J, L are higher magnifications of A, C, E, G, I, K, respectively. Five to six animals were used for each experimental group. The dotted lines indicate the SNpc. Scale bars, 250 µm. M and N: Detection for protein oxidation reveals that CB1 receptor reduces the protein carbonyls in MPTP-treated SN. Samples were analyzed by Western blotting for protein carbonyls as markers of oxidatively modified proteins. N: Bars represent the means ± SEM of four to five samples. *p < 0.01 compared with control, **p < 0.01, ***p < 0.05, significantly different from C; # # # p < 0.05, significantly different from M; ¤ ¤ ¤ p < 0.01, significantly different from MPTP; MH, MPTP and HU210; MWA, MPTP, HU210, and AM251; MW, MPTP and WIN-55,212-2; MWA, MPTP, WIN-55,212-2, and AM251.

WIN55,212-2 and HU210 protect DA neurons from microglia-derived neurotoxicity

Our results showed that the in vivo neuroprotective effects of nonselective cannabinoid receptor agonists WIN55,212-2 and HU210 are not attributable to reduced metabolism of MPTP to MPP⁺ in DA neurons (Table I). However, the possibility remained that WIN55,212-2 and HU210 might promote neuronal survival by preventing MPP⁺-induced blockade of mitochondrial respiration in neurons. To test this hypothesis, additional experiments were performed in mesencephalic neurons cultured alone or cocultured with microglia. In microglia-free, neuron-enriched mesencephalic cultures, pretreatment with 0.1–1.0 µM WIN55,212-2 and HU210 (30 min before MPP⁺ treatment) had no protective effect (Fig. 7A–E, 7K), and 3 µM WIN55,212-2 and HU210 alone was toxic (data not shown). These results carefully suggested that this observed neuroprotection is not due to prevent either MPP⁺-induced blockade of mitochondrial respiration in neurons or MPP⁺ uptake into DA neurons.

By contrast, in cocultures of mesencephalic neurons and microglia, WIN55,212-2 and HU210 (0.5–1.0 µM) blocked MPP⁺-induced death of DA neurons (Fig. 7F–L, suggesting that WIN55,212-2 and HU210 act through microglia to mediate its neuroprotective effects. Moreover, pretreatment with AM251 (2 µM), a CB1 receptor antagonist, reduced the number of TH-positive cells observed with 1 µM WIN55,212-2 (45%; p < 0.01; Fig. 7L) and 1 µM HU210 (48%; p < 0.01; Fig. 7L) in MPP⁺-treated cocultures of mesencephalic neurons and microglia, indicative of microglia CB1 receptor involvement (48).

Discussion

Accumulating evidence suggests that the cannabinoid system is a promising pharmacological target for the treatment of PD (49, 50) and levodopa-associated motor complications (51, 52). The CB1 receptor regulates motor behavior in the basal ganglia via mediating both excitatory and inhibitory inputs to the SN reticulate and globus pallidus (53) as well as short- and long-term synaptic plasticity through suppressing the release of neurotransmitters, such as glutamate and GABA (54, 55). Additionally, cannabinoids modulate inflammatory responses by regulating microglial function through both receptor-dependent and -independent mechanisms (39, 56–58).

Microglia, resident immunocompetent and phagocytic cells in the CNS, play a critical role in innate defense (59). These intrinsic
FIGURE 7. Effects of cannabinoids on MPP⁺-induced neurotoxicity in mesencephalic cultures. A and F are phase-contrast optics of B and G, respectively. Arrows indicate presence of microglia. TH-positive neurons had long and branched neuritic processes, indicating protective effect of WIN55,212-2 and HU210 against MPP⁺ neurotoxicity in cocultures, many of the remaining TH-positive neurons had short processes and rounded and shrunken cell bodies compared with vehicle-treated control. These results clearly indicate that WIN55,212-2 and HU210 have no effect against MPP⁺-induced neurotoxicity in microglia-free neuron-enriched mesencephalic cultures. TH-positive neurons in cocultures of mesencephalic neurons and microglia treated with vehicle as a control (G) and 10 μM MPP⁺ for 48 h in the absence (H) or presence of WIN55,212-2 (1 μM) (I) and HU210 (0.1–1 μM) (J) pretreatment for 30 min. Note that following either MPP⁺ or MPP⁺ plus WIN55,212-2/HU210, several of the remaining TH-positive neurons displayed short processes and rounded and shrunken cell bodies compared with the vehicle-treated control. These results clearly indicate that WIN55,212-2 and HU210 have no effect against MPP⁺-induced neurotoxicity in microglia-free neuron-enriched mesencephalic cultures. TH-positive neurons in cocultures of mesencephalic neurons and microglia treated with vehicle as a control (G) and 10 μM MPP⁺ for 48 h in the absence (H) or presence of WIN55,212-2 (1 μM) (I) and HU210 (0.1–1 μM) (J) pretreatment for 30 min. Similar to those observed in neuron-enriched cultures (B–E), following MPP⁺ treatment, many of the remaining TH-positive neurons had short processes and rounded and shrunken cell bodies compared with vehicle-treated control. By contrast, following WIN55,212-2 and HU210 treatment, TH-positive neurons had long and branched neuritic processes, indicating protective effect of WIN55,212-2 and HU210 against MPP⁺ neurotoxicity in cocultures. Scale bar, A–J, 50 μM. K. TH-positive neurons were counted. L. Number of TH-positive neurons in cocultures of mesencephalic neurons and microglia treated with MPP⁺ in either the absence or presence of WIN55,212-2 or HU210 and CB₁ receptor antagonist. Cultures were pretreated with 2 μM AM251 for 30 min before treatment with 1 μM WIN55,212-2 or HU210 for 48 h. All values are expressed as means ± SEM of triplicate cultures from three separate plates. *p < 0.001, **p < 0.001, compared with each control values; #p < 0.01, compared with MPP⁺ only-treated cocultures; $p < 0.05, compared with MPP⁺ only-treated cocultures; $p < 0.01, compared with MPP⁺- and WIN55,212-2-treated cocultures; $p < 0.01, compared with MPP⁺- and HU210-treated cocultures (ANOVA and Student–Neuman–Keuls analysis).
in cultured mouse cerebellar granule cells devoid of microglia, as assessed using the Live and Dead assay. The apparent discrepancy between the results obtained from these two studies may be attributed to differences in toxin (MPP+ versus 6-OHDA) and cell types (rat mesencephalic neurons versus mouse cerebellar granule neurons).

In this study, we have shown that WIN55,212-2 and Hu210 prevent DA neuronal death in the MPTP model of PD through inhibition of microglial activation, which can be reversed by CB1 receptor selective antagonists AM251 and SR14716A, clearly indicative of CB1 receptor involvement. However, these results may be irrelevant for CB1 receptor-independent neuroprotection in the MPTP model (42). WIN55,212-2 rescued nigrostriatal DA neurons and inhibited microglial activation in CB1 receptor knock-out mice treated with MPTP, indicating CB1 receptor-independent activity. The inconsistent experimental results between the two studies are probably a result of the different methods used for CB1 receptor inhibition (genetic ablation of CB1 receptor versus activity). The inconsistent experimental results between the two studies in MPTP-treated mice, indicating possible involvement of microglial activation and microglia-derived oxidative damage.

These results cannot necessarily rule out the possibilities that activation of CB1 receptor is in keeping with the present biochemical and behavioral evidence that MPTP induces depletion of dopamine in the STR, with resultant motor dysfunction. Importantly, activation of the CB1 receptor inhibits microglial activation-mediated oxidative stress, suggest that the CB1 receptor is a useful pharmacological target for treating PD and other disorders associated with neuroinflammation and microglia-derived oxidative damage.

Disclosures
The authors have no financial conflicts of interest.

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