Blockade of Cannabinoid CB1 Receptors Augments the Antiparkinsonian Action of Levodopa without Affecting Dyskinesias in MPTP-Treated Rhesus Monkeys

Xuebing Cao, Li Liang, John R. Hadcock, Philip A. Iredale, David A. Griffith, Frank S. Menniti, Stewart Factor, J. Timothy Greenamyre, Stella M. Papa

Department of Neurology, Emory University, Atlanta, GA
Department of Neurology, Emory University. Atlanta, GA (XC, LL, SF and SMP)
Department of Neurology, University of Pittsburgh, Pittsburgh, PA (JTG)
Neuroscience Biology, Pfizer Global Research & Development. Groton, CT (JRH, PAI and FSM)
CVMD Chemistry, Pfizer Global Research & Development. Groton, CT (DAG)
Running Title: Blockade of CB1 receptors potentiates L-DOPA effects

Corresponding author: Stella M. Papa, M.D. Department of Neurology, Emory University. 6000 WMRB, 101 Woodruff Circle. Atlanta, Georgia 30322, US.
Tel:+1-404-727-8307(O), Fax:+1-404-727-9294, E-mail: spapa@emory.edu

Total number of text pages: 40

Number of Tables: 3
Number of figures: 6
Number of references: 40

Number of words in Abstract: 242
Number of words in Introduction: 709
Number of words in Discussion: 1094

Abbreviations: CE, 1-[7-(2-Chlorophenyl)-8-(4-chlorophenyl)-2-methylpyrazolo[1,5-a]-[1,3,5]triazin-4-yl]-3-ethylaminoazetidine-3-carboxylic acid amide benzenesulfonate; CP-55940 [(1R,3R,4R)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol]; L-DOPA, L3,4-dihydroxyphenylalanine; CB1, Cannabinoid type 1 receptor; 2AG, 2-arachidonylglycerol; GABA, gamma-aminobutyric acid; 5-HT1B, serotonin receptor 1B; GPe, globus pallidus external segment; GPi, globus pallidus internal segment; SNr, substantia nigra pars reticulata; PD, Parkinson’s disease; LID, L-DOPA-induced dyskinesias; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; HPLC, High-
performance liquid chromatography; ED, electrochemical detection; ANOVA, analysis of variance.

**Recommended section assignment**: Neuropharmacology
Abstract

Drugs acting at cannabinoid CB1 receptors have modulatory effects on glutamate and GABA neurotransmission in basal ganglia, and thereby potentially affect motor behavior in the parkinsonian setting. Preclinical trials with diverse cannabinoid agents have shown varied results, and the precise effects of blocking cannabinoid CB1 receptors remain uncertain. We tested behavioral effects of the selective antagonist, CE, as monotherapy and in combination with L-DOPA in treatment-naïve and L-DOPA-primed MPTP-treated rhesus monkeys with moderate and severe parkinsonism. Motor disability and L-DOPA-induced dyskinesias were scored with a standardized scale after subcutaneous drug administration, and plasma levels of L-DOPA were determined by HPLC/ED. CE doses ranged from 0.03 to 1 mg/kg, and L-DOPA methyl ester doses were selected as optimal and suboptimal doses (maximal and 50% of maximal responses, respectively). CE had no intrinsic effects on motor behavior regardless of the degree of parkinsonism (moderate or severe groups) or previous drug exposure (‘de novo’ or after L-DOPA priming). Initial CE administration did not affect development of L-DOPA antiparkinsonian responses. In co-administration trials, CE in a dose-dependent manner increased responses to L-DOPA (suboptimal doses). These effects were seen in both moderate and severely parkinsonian monkeys as a 30% increase of, predominantly, response duration with no effects on L-DOPA pharmacokinetics. CE did not modify levodopa-induced dyskinesias. These results suggest that selective cannabinoid CB1 antagonists may enhance the antiparkinsonian action of dopaminomimetics, and possibly facilitate the use of lower doses, thereby reducing side effects.
Introduction

Parkinson’s disease (PD) is characterized principally by progressive neurodegeneration of the nigrostriatal dopamine system and its accompanying motor dysfunction; tremor, rigidity and bradykinesia. Dopamine replacement with the dopamine precursor L-DOPA improves motor symptoms, although long-term therapy causes disabling side-effects, such as varied motor complications (response fluctuations and dyskinesias) (Nutt, 2000; Obeso et al., 2000). Because of these shortcomings, therapies that act as either adjuncts or alternatives to L-DOPA by modulating its effects and reducing adverse reactions may help restoring normal function in the late-stage disease. Putative bases for developing new therapies lie in the interaction of dopamine with other neurotransmitter systems in basal ganglia. In fact, pathogenic mechanisms of L-DOPA-induced motor complications involve the glutamate system as the major transmitter driving the activity of striatal neurons (Chase and Oh, 2000). Other neurotransmitters may also play a role, and in recent years, non-dopaminergic symptomatic therapies have been extensively sought for ameliorating L-DOPA motor complications (Papa and Chase, 1996; Grondin et al., 1999; Papa et al., 2004).

The cannabinoid system appears to have important influences in dopamine-mediated mechanisms within basal ganglia (Di Marzo et al., 1998). Cannabinoid CB1 receptors and the endocannabinoids, anandamide and 2-arachidonyl glycerol (2AG) (Herkenham et al., 1990; Di Marzo et al., 2000) are particularly abundant in striatum and the striatal terminals in the globus pallidus (GP in rodents; GPe or globus pallidus external segment in primates) and substantia nigra pars
reticulata/internal globus pallidus (SNr/GPi), the “indirect” and “direct” striatal output pathways, respectively. In striatum, presynaptic CB1 receptors regulate glutamate release and reuptake, and the combination of these effects results in reduction of glutamate-mediated postsynaptic excitation (Gerdeman and Lovinger, 2001; Brown et al., 2003). In addition, striatal CB1 receptors seem to regulate dopaminergic and serotoninergic (5-HT1B) signaling (Hermann et al., 2002), and increased binding of CB1 receptors and activity of G protein coupling have been demonstrated in the striatum of MPTP-treated marmosets and PD patients (Lastres-Becker et al., 2001). In the GPI/SNr the net effects of CB1 stimulation are uncertain because of combined inhibitory interaction with GABA release and uptake from striatal terminals as well as glutamate release from the subthalamic nucleus (STN) (Szabo et al., 2000; Wallmichrath and Szabo, 2002). However, in GPe, the main effects are thought to be mediated by inhibition of GABA re-uptake that results in increased GABA transmission and inhibition of the STN (Maneuf et al., 1996). Through this mechanism in the indirect striatal output pathway, CB1 antagonists may synergize the L-DOPA antiparkinsonian effects. Nevertheless, the effects of cannabinoid agonists and antagonists after systemic administration remain unclear due to their complex actions in multiple basal ganglia sites.

Earlier studies of cannabinoid drugs in rodents have shown marked motor effects, although data derived from agonists and antagonists have not correlated with findings of equivalent tests in primates (Meschler et al., 2000a; Meschler et al., 2000b). Studies of CB1 receptor antagonists in non-human primates have reported contradictory results (Di Marzo et al., 2000; Meschler et al., 2001; van der Stelt et
al., 2005), whereas in clinical trials they did not show positive effects (Mesnage et al., 2004). Recently, the selective CB1 antagonist, rimonabant, was shown to have antiparkinsonian actions per se and to reduce L-DOPA-induced dyskinesias (LID) in MPTP-treated primates (van der Stelt et al., 2005). These data suggest that CB1 receptor-mediated transmission plays a functional role in different motor behaviors developed in the chronic course of the disease. However, the effects of selective CB1 receptor antagonists have not been studied thoroughly in primates.

In this study, a selective cannabinoid CB1 antagonist, CE (Figure 1), was used for a series of trials in parkinsonian monkeys. CE is a highly selective full antagonist for the CB1 receptor that is well absorbed and readily gains access to the brain. We used MPTP-treated rhesus monkeys in the following CE trials: 1-monotherapy for dose response curves, 2-monotherapy for comparison with L-DOPA as ‘de novo’ treatments in a crossover design trial (also CE effects were tested before and after L-DOPA priming), 3-co-administration with L-DOPA in moderately parkinsonian monkeys, 4-co-administration with L-DOPA in severely parkinsonian monkeys with LID, and 5-determination of CE interaction with L-DOPA pharmacokinetics in tests of co-administration. These trials produced the data for a complete behavioral profile of CB1 antagonists in parkinsonian primates.
Methods

Subject Preparation

Seven adult monkeys (Macaca Mulatta), 2 males and 5 females, between 5 and 8 kg of weight, were kept in controlled housing conditions with constant temperature, relative humidity, and 12 h light/dark cycles. Animals had free access to food, fresh fruit supplements, and water. All studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and approved by the Institutional Animal Care and Use Committee. Monkeys received MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, 0.5 to 0.8 mg/kg i.v.) once weekly for 6 months or longer until they had a stable parkinsonism of moderate to severe degree. Four new animals were rendered moderately parkinsonian for this study to compose the ‘de novo’ group. The other three monkeys were severely parkinsonian, received L-DOPA as regular treatment before entering this study, and formed the group of dyskinetic monkeys. L-DOPA was given orally (25 to 50 mg, Sinemet® 25/100) twice daily until development of consistent and reproducible dyskinesias. Motor disability was scored using a standardized scale for parkinsonian primates (see below), and scores are shown in Table 1.

CE Pharmacological Characterization

1-CB1 and CB2 receptor binding. HEK293 (CB1) or CHO (CB1 and CB2) cells (ATCC) were stably transfected with the human CB1 or CB2 receptors, and membranes were prepared as described (Bass et al., 1996). A Pierce BCA kit was used to determine protein concentrations. CE was diluted in buffer (25 mM Tris, 5 mM MgCl2, 1 mM EDTA, pH 7.4) containing 0.5% BSA, 10% DMSO, and then 25 µl of these solutions
were added to 96 well polypropylene plate. [3H] SR141716A (1.2 nM final concentration) was diluted in a buffer containing 0.5% BSA, and 25 µl were added to the plate. The plates were covered and placed in an incubator at 30 °C for 60 minutes. The reaction was stopped by addition of 125 µl of buffer containing 10% BSA, and then membranes were collected onto GF/C filter plates (Perkin Elmer) presoaked in buffer containing 0.5% BSA. Filters were washed twice and then dried overnight. Filters were counted on a Wallac Trilux™ counter. CB2 receptor binding was assayed under the same conditions but using [3H]CP-55940 (10 nM final concentration) as radioligand. 2-GTPγ[35S] binding assays at CB1 receptors.

GTPγ[35S] binding assays were performed in a 96 well FlashPlate™ format in duplicate using 100 pM GTPγ[35S] and 10 µg membrane per well in assay buffer composed of 50 mM Tris HCl, pH 7.4, 3 mM MgCl₂, pH 7.4, 10 mM MgCl₂, 20 mM EGTA, 100 mM NaCl, 30 µM GDP, 0.1% bovine serum albumin and protease inhibitors (100 µg/ml bacitracin, 100 µg/ml benzamidine, 5 µg/ml aprotinin, 5 µg/ml leupeptin). The assay mix was incubated with increasing concentrations of CE (10⁻¹⁰ M to 10⁻⁵ M) for 10 minutes and challenged with the cannabinoid agonist CP-55940. Assays were performed at 30 °C for one hour. The FlashPlates™ were then centrifuged at 2000Xg for 10 minutes. Stimulation of GTPγ[35S] binding was then quantified using a Wallac Microbeta. 3-Cannabinoid Tetrad. Cannabinoids have long been associated with inducing a set of four, well-characterized, centrally mediated behaviors in rodents: hypothermia, anti-nociception, hypolocomotion and ring immobility (catalepsy), known as the in vivo tetrad (Little et al., 1988). The efficacy and potency of CE to reverse the effects mediated by the agonist CP-55940 were
examined in groups of male mice (17-19 g). Animals were given vehicle or CE (0.3, 1.0 or 3.0 mg/kg s.c.) followed 15 min later by vehicle or CP-55940 (0.78 mg/kg s.c.). Twenty-five min after CP-55940 administration, mice were placed in acrylic cages, and activity was recorded for 5 min using infrared motion detectors (Coulbourn Instruments) placed on top of the cage. Immediately afterwards, animals were placed on a hot plate apparatus (Columbus Instruments), and we recorded the latency to flick or lick a hind paw, or jump from the hot plate, after which animals were removed (40 second cut off). Twenty min after the hot plate test, temperatures (to the nearest tenth of a degree) were recorded using a small thermostat probe inserted 2 - 2.5 cm into the rectum. Fifteen min later, catalepsy was assessed by placing animals on a horizontal 6.5 cm steel ring attached to a ring stand at a height of 12 inches. The animal was suspended in the gap of the ring with fore and hind paws gripping the perimeter. The time of remaining completely motionless (except for respiratory movements) was recorded over a 3-min period. An immobility rating was calculated as the percentage of the motionless period from the total time of observation.

In vitro pharmacologic profiling showed that CE exhibits both high affinity binding to and functional antagonism of the human CB1 receptor expressed in CHO cells. CE displaces binding of [³H]SR-141716A to the human CB1 receptor with a Ki of 0.33 nM. In contrast, the compound has a lower affinity for the human CB2 receptor with a Ki = 10,000 nM that indicates a 30,000-fold selectivity. CE is also 2,000-fold selective for CB1 receptor binding over a panel of 53 receptors, ion channels, and uptake sites expressed in the CNS. Following agonist binding to the CB1 receptor,
G protein activation occurs, and GTP or analogs bind the receptor complex with high affinity. Thus, CB1 agonist induced-increase in GTP\(\gamma\)[\(^{35}\)S] binding was used as a measure of CB1 receptor activation, and inhibition of GTP\(\gamma\)[\(^{35}\)S] binding was used to measure the antagonist potency and efficacy of CE. CE blocked CB1 agonist CP-55,940-stimulated GTP\(\gamma\)[\(^{35}\)S] binding with a Ki = 0.07 nM. Inhibition was surmountable and of a mixed competitive/non-competitive type (K\(B\) = 0.63 nM, slope = 5.8). In vivo CE testing in the cannabinoid tetrad confirmed central CB1-mediated effects. CE dosed at 0.3 mg/kg, s.c., significantly reversed the effects of a centrally acting cannabinoid agonist (CP-055,940; 0.78 mg/kg, i.p.) in three of the four components of the tetrad, and all four at 1 mg/kg (Table 2).

**Drugs**

CE (1-[7-(2-Chlorophenyl)-8-(4-chlorophenyl)-2-methylpyrazolo[1,5-a]-[1,3,5]triazin-4-yl]-3-ethylaminoazetidine-3-carboxylic Acid Amide Benzenesulfonate) is described in US Patent Publication No. 2004/0157839 (Figure 1). Human CB1 and CB2 receptor cDNAs and/or cell lines were the gift of Dr. Debra Kendall (University of Connecticut). CP-55940 [(1R,3R,4R)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol] was synthesized at Pfizer, Global Research and Development (Groton, CT). \([^3\text{H}]\text{CP55,940\ (158 Ci/mmol)}\) and GTP\(\gamma\)[\(^{35}\)S] were purchased from Perkin Elmer Life Sciences (Boston, MA). \([^3\text{H}]\text{SR141716A\ (44.0 Ci/mmol)}\) was purchased from Amersham Pharmacia (Piscataway, NJ). L-DOPA methyl ester, benserazide and MPTP were purchased from Sigma-Aldrich (St. Louis, MO, US). Oral carbidopa/levodopa (generic Sinemet® 25/100 mg) was purchased from Henry Schein (Denver, PA, US). Cavasol was supplied from Wacker Chemical
Corp. (Adrian, MI, US) and dissolved in distilled water (30% cavasol solution). CE was dissolved completely at a concentration of 0.6 mg/ml in Cavasol solution. L-DOPA methyl ester and benserazide were dissolved in saline.

**Experimental Drug Tests**

1. **CE dose-response curve**

To determine the intrinsic effects of CE on parkinsonian motor symptoms, we tested CE as monotherapy at different doses (dose-response curve) in the group of three severely parkinsonian monkeys. At the time of testing, animals had a stable and chronic parkinsonism. Also, they had been treated regularly with oral L-DOPA, which was withdrawn for 48 hours prior to each CE test.

   All tests were performed at mid-morning time. In every test, monkeys received two immediately successive injections, the first injection was a certain dose of CE, and the second injection was always L-DOPA vehicle (for subsequent comparisons with L-DOPA effects if CE had intrinsic effects). We tested four doses of CE: 0.03, 0.1, 0.3, and 1 mg/kg, and all injections were given subcutaneously. Thus, CE tests were: a-CE vehicle + L-DOPA vehicle; b-CE 0.03 mg/kg + L-DOPA vehicle; c-CE 0.1 mg/kg + L-DOPA vehicle; d-CE 0.3 mg/kg + L-DOPA vehicle; e-CE 1 mg/kg + L-DOPA vehicle. Each test was repeated at least once, and results from two or three tests were averaged to generate each animal data points for statistical analysis.

2. **Comparison of CE and L-DOPA as ‘de novo’ treatments**

In this part, we used the group of four monkeys that had a milder parkinsonism and were naïve of antiparkinsonian treatment. One aim of this study was to detect any discrete intrinsic effects of CE that could not be identified in tests of severely
impaired monkeys. Another aim was to determine if previous development of antiparkinsonian responses with L-DOPA induced CE effects as a priming mechanism that has been described with other agents. To accomplish these aims, the study had a crossover design that compared the effects of CE and L-DOPA as ‘de novo’ treatments, i.e. two treatment series that were switched across two groups of monkeys (group 1, monkeys A and B; group 2, monkeys C and D). Treatment series 1: first injection CE vehicle, and immediate second injection one of the two doses of L-DOPA (LD-low or LD-high). Treatment series 2: first injection any of the two doses of CE (0.3 or 1 mg/kg), and immediate second injection L-DOPA vehicle. Table 3 shows the crossover sequences; monkeys A and B were tested first with L-DOPA (Treatment series 1) and subsequently they were tested with CE (Treatment series 2); monkeys C and D started with CE tests and then switched to L-DOPA tests. Each test was repeated at least once, and results of two or three tests were averaged. Several weeks elapsed in between the two treatment series, so there were no residual effects of CE or L-DOPA. CE doses were selected from the highest doses of previous tests where CE had no intrinsic effects (dose-response curve), i.e.: 0.3 and 1 mg/kg. L-DOPA doses were selected as an “optimal” dose, the minimal dose that produced maximal effects (LD-high, usually 100–200 mg), and a “suboptimal” dose, the dose that produced considerably lower but unequivocally measurable effects (LD-low, usually 50–100 mg). These two doses of subcutaneous L-DOPA methyl ester plus benserazide were determined at the time of beginning L-DOPA testing in each group.

3. Co-administration of CE and L-DOPA
In this part, we tested the effects of CE on the antiparkinsonian responses to L-DOPA in both groups, moderately (one monkey was not included because of unstable responses to L-DOPA) and severely parkinsonian monkeys. Optimal and suboptimal L-DOPA doses were selected as described above. In the moderately parkinsonian group, the two highest doses of CE (0.3 and 1 mg/kg) were co-administrated with L-DOPA. In the severely parkinsonian group, three doses of CE were used to include a lower dose for full assessment of effects on dyskinesias (0.1, 0.3, and 1 mg/kg). Vehicle injections were also tested here to update the control results. In these tests, animals received: first, CE vehicle, 0.1, 0.3, or 1 mg/kg, and second, LD-low or LD-high. All tests had immediately successive injections. Tests were repeated at least once, and they were separated by intervals of three days at minimum as drug washout periods. Results are the average of two or three experiments.

Behavioral Assessment

Motor behavior was assessed with a standardized Motor Disability Scale developed for MPTP-treated primates that has two parts, Part I-Motor Disability (items are presented in Table 1) and Part II-Drug-Induced Adverse Reactions (Papa and Chase, 1996). Motor behavior is itemized in the scale according to expression of parkinsonian symptoms and, thus, the scale's sensitivity is higher than most motor tasks in parkinsonian macaques. In addition, this type of scale was developed for macaques as equivalent to the rating scales utilized in clinical trials for patients. Scores were taken just before drug injections (time 0) and afterwards starting at 30 minutes and continuing every 20 min intervals until there was a return to baseline.
Dyskinesias were assessed at the same time points using Part II of the scale that rates dyskinesias with a wide range within the following major categories: 0 = absent, 1 = mild (fleeting, rare, present less than 30% of the observation period), 2 = moderate (interfering with normal activity, present less than 60% of observation period), and 3 = severe (disabling, replacing normal activity, present more than 90% of observation period). All tests were performed after an overnight fast. Animals were videotaped for subsequent rating by a blinded investigator. Each test in all sets of experiments was repeated. Data were averaged to yield a mean from two to three data points for each treatment in each monkey.

**Plasma Levels of L-DOPA**

To determine CE interactions with L-DOPA pharmacokinetics, the plasma levels of L-DOPA were assessed when co-administrated with CE at the highest dose of CE tested in these studies (1 mg/kg) in two chair-trained monkeys. L-DOPA, at the dose whose effects were modified by the addition of CE (LD-low, 75 mg), was administered immediately following the CE injection. Both drugs were administered subcutaneously as in previous tests. Blood samples were collected beginning with time 0 for baseline (before drug administration), and thereafter at 45, 90, 105, 120, 135, and 150 minutes. In each monkey, blood samples were taken in 3 repeated experiments for control treatment (CE vehicle + LD-low) and 3 repeated experiments for CE treatment (CE 1 mg/kg + LD-low). Whole blood was centrifuged and separated plasma samples were stored at -80°C until analysis. L-DOPA levels were determined based on modification of previously published methods (Blandini et al., 1997). Briefly, plasma was deproteinized by addition of equal volume of 1.2 M
HClO₄ and then diluted 1:5 in H₂O. After centrifugation for 30 minutes (15,000 rpm at room temperature), the supernatant was collected for analysis by HPLC with ED. Ten µl of supernatant were applied to an in-line pre-oxidation electrode (ESA 5020 guard cell electrode set at 0.20V, ESA Inc., Chelmsford, MA) followed by a reverse-phase C18 Thermo (Thermo Fisher Scientific Inc., Waltham, MA) column (150 × 3 mm, 3 µm particle size, and pore size 120a). The column was eluted with an aqueous mobile phase consisting of 50 mM KH₂PO₄, 0.7 mM SDS, 0.3 mM EDTA, containing 12% acetonitrile (pH = 2.9 with glacial acetic acid). Mobile phase flow rate was 0.200 ml/min, controlled by a Shimadzu LC-10AD pump (Shimadzu USA Manufacturing Inc., Camby, OR). L-DOPA was detected in the column effluent using an ANTEC electrochemical detector (ANTEC Leyden, The Netherlands) with a working electrode potential of 0.30V. Data were processed by Empower Pro 2 software from Waters Corporation (Milford, MA). L-DOPA level in each sample was determined from the peak height in comparison to a curve generated using 3 concentrations of authentic L-DOPA as standard. Plasma samples are expressed as ng/ml.

Statistical Analysis

Scores of motor disability and dyskinesias were graded within wide ranges, and thus, data composed continuous variables. All results are expressed as mean values ± S.E.M. Two-factor analysis of variance (ANOVA) for repeated measures, followed by the post hoc Fisher’s Protected least significant difference (PLSD) test when the f indicated significance, were used to compare every treatment over the time course after drug administration. Significance was taken at $p < 0.05$. 

-16-
Results

Intrinsic Effects of CE

CE as monotherapy had no measurable effects on parkinsonian monkeys with severe motor impairment. Motor disability scores remained unchanged during the two hours after administration of every CE dose. Scores averaged 15.5 ± 2.5, 15.2 ± 2, 15.1 ± 2, 15.1 ± 2, and 15.6 ± 2.2 after CE vehicle, 0.03, 0.1, 0.3, and 1 mg/kg, respectively (p > 0.05). While monkeys had a tendency to relax, motor disability was not worsened (unchanged scores in the scale). Monkeys did not exhibit retching, vomiting, or changes in social interaction. The highest doses of CE produced slight somnolence in two animals. This effect was observed irregularly, and it did not affect the animal’s interaction with the examiner.

CE was also tested as monotherapy in moderately parkinsonian monkeys during the study of comparison with L-DOPA (crossover). CE per se did not have motor effects in this group of monkeys either. Since in this study CE monotherapy was also evaluated after monkeys received L-DOPA tests, results show that monotherapy with CE had no effects in treatment-naïve or L-DOPA-primed monkeys, i.e.: responses to CE did not change between the groups (Table 3). Monkeys that received CE treatment after developing antiparkinsonian responses to L-DOPA had no antiparkinsonian response to CE, just as monkeys that received CE treatment initially. L-DOPA and CE responses were clearly different (p < 0.001, Table 3). On the other hand, the antiparkinsonian effects of L-DOPA before and after CE administration were similar in all animals. Therefore, CE did not potentiate initial responses to L-DOPA. Nor did it modify the course of development and
stabilization of L-DOPA responses. Altogether, these results indicated that CE had no intrinsic effects on motor behavior of parkinsonian monkeys.

**Effects of CE on Antiparkinsonian Action of L-DOPA**

Co-administration of CE with L-DOPA resulted in enhanced antiparkinsonian effects. Responses to the suboptimal dose of levodopa were prolonged by the addition of CE in parkinsonian monkeys with different degrees of disability. In the severely parkinsonian group, a significant effect (CE + LD-low) was found on motor disability scores accumulated over the whole antiparkinsonian response, total scores (Figure 2A). These effects were dose dependent, and they were clearly manifested at the two higher doses, 0.3 and 1 mg/kg (p < 0.05 compared with baseline response of L-DOPA low dose, CE vehicle + LD-low). The reduction of total disability scores by adding CE derived from extended duration of low scores as shown in figure 2B (p < 0.01 for differences of scores at late individual intervals). Prolongation of L-DOPA responses varied among animals from 20 to 40 minutes after co-administration of CE, and this time represents 30% increase of response duration for L-DOPA low doses, which average 100 minutes. Co-administration of CE with L-DOPA optimal doses resulted in slighter effects in severely parkinsonian monkeys suggesting that a maximal effect was reached (difference of scores at individual time points did not attain significance). The minimal scores obtained at the peak of L-DOPA effects (LD-low and LD-high) did not significantly change with the addition of any dose of CE.

In the moderately parkinsonian group, a significant effect of CE in combination with LD-low was also found on total motor disability scores accumulated during the
antiparkinsonian response ($p < 0.01$, Figure 3A). The addition of CE at the highest dose (1 mg/kg) had a tendency to reduce motor disability to lower scores at the peak effect and extended the reversal of parkinsonism from 80 to 110 min (Figure 3B). L-DOPA high doses produced a marked response in this group that remained unchanged by the addition of CE.

On average from all parkinsonian monkeys, a substantial difference between treatments is demonstrated at 110 minutes from the injections, and it is maintained at 130 minutes. Figure 4 shows changes of response duration with co-administration of CE and LD-low in every monkey. Scores are also lower during the peak effect with the combination of CE 1 mg/kg and LD-low, although these differences were inconsistent.

**Effects of CE on L-DOPA-Induced Dyskinesias**

CE had no effects on L-DOPA-induced dyskinesias. Severely parkinsonian monkeys had stable dyskinetic responses to L-DOPA. Dyskinesias were typically peak-dose and of choreic type, although dystonic dyskinesias were also seen with the tested doses of L-DOPA. Mostly neck and trunk dystonia together with choreic movements of the limbs were present with the higher doses of L-DOPA. Dystonia was seen in the legs less frequently. Figure 5 shows scores of dyskinesias in each interval for all treatment combinations of CE and L-DOPA. A slight difference (non-significant) in values is present in the late intervals, and thus, this effect is likely related to the prolongation of levodopa action by the addition of CE. The peak values of dyskinesias were not changed with the addition of CE. CE had no
significant effect on individual scores of dyskinesias at any given time point (Figure 5A and B).

**Plasma Levels of L-DOPA**

CE had no effects on L-DOPA pharmacokinetics. Following systemic administration of L-DOPA plus benserazide at the suboptimal doses, L-DOPA concentration in plasma increased steadily until reaching its peak at 45 min, and then it gradually decreased during the remaining 100 minutes of behavioral effects (Figure 6). Curves of plasma levels of L-DOPA were similar following co-administration of CE vehicle and the highest CE dose (1mg/kg) that prolonged L-DOPA responses. No significant differences in plasma concentrations of L-DOPA were found in samples taken at any time during repeated determinations in two monkeys (Figure 6). In fact, plasma levels of L-DOPA were slightly lower after administration of CE than after vehicle. Thus, the effects of CE on prolonging the response to L-DOPA were not due to prolongation of L-DOPA plasma concentration.
Discussion

Results of this study demonstrate that CE potentiates the antiparkinsonian action of levodopa in MPTP-treated rhesus monkeys. CE in a dose-dependent manner augmented L-DOPA responses by lowering scores of motor disability at the peak effect, and more markedly, by prolonging effects by 30% of the usual duration after a suboptimal dose of L-DOPA. This prolongation of L-DOPA response was not related to pharmacokinetic interactions because plasma levels of L-DOPA were not affected by CE co-administration. CE effects are, thus, mediated by its central action at the CB1 receptors where this drug exerts full antagonism.

These data originally show that blockade of CB1 receptors augments the antiparkinsonian effects of L-DOPA in the rhesus monkey. The study also shows that CB1 receptor antagonism has: 1-no intrinsic antiparkinsonian effects, and 2-no antidyskinetic effects. Previous studies of cannabinoid agonists and antagonists have shown conflicting results where, for instance, antagonists were found to be effective as monotherapy and to have antidyskinetic effects (Fernandez-Espejo et al., 2005; van der Stelt et al., 2005). Because we used MPTP-treated macaques, that closely mimic motor behavior and basal ganglia anatomy of humans, and the inclusive experimental design from monotherapy to multiple aspects of levodopa co-administration, this study provides critical data to clarify the potential role of CB1 antagonists in the therapy of PD. Most previous work in primates derives from trials with the CB1 antagonist rimonabant (SR141716A) in marmosets. Despite a homologous neuroanatomy (Hardman et al., 2002), definite differences between these non-human primate species are evidenced by the sensitivity to MPTP and the
responsiveness to L-DOPA. In marmosets, a stable parkinsonism is rapidly produced (5 days), and dyskinesias are induced after short drug exposure (10 days) (Pearce et al., 1995), as opposed to several months of both MPTP and L-DOPA treatments required in macaques. Additionally, different motor behavior of marmosets (high baseline activity) is commonly assessed with locomotor activity and range of movement. Results from these measurements are not equivalent to our scoring system using standardized scales that were designed to quantify specific parameters of parkinsonian disability, such as tremor, posture, stability, etc. Therefore, this study presents unequivocal data that demonstrate potentiation of L-DOPA antiparkinsonian effects by CB1 antagonists. These drugs have barely been tested in parkinsonian patients by limited trials of a single challenge with a single dose (Mesnage et al., 2004). Our results support a thorough clinical evaluation of CB1 antagonists.

CE had antiparkinsonian effects in our two groups of monkeys that differed not only in their motor impairment, but also in their responsiveness to L-DOPA. High sensitivity in severe conditions leads to a similar high effect close to a ‘ceiling’ effect with different doses of L-DOPA (‘all or none’ response) (Nutt and Holford, 1996). Severely parkinsonian monkeys also have motor fluctuations typically characterized by shortened responses, and they may have a reduced capability to adjust the response duration to different doses (Nutt, 2001). Thus, motor responses to dopamine in the advanced stages of the disease involve altered dopamine-mediated mechanisms, and results of CE in both conditions of parkinsonism suggest a
cannabinoid mechanism that is independent of direct interaction with a certain
dopamine signaling.

Lack of intrinsic effects of CB1 antagonists was also found in cynomolgus
monkeys with rimonabant (Meschler et al., 2001). Here, CE was tested in monkeys
naïve of antiparkinsonian treatment to reveal slighter motor effects that are
overshadowed after development of full responses to dopamine stimulation in
conditions of marked motor impairment (Nutt and Holford, 1996). Also, CE was
tested in monkeys that had been primed with dopaminergic stimulation, which can
induce a response to drugs acting on other systems (Morelli et al., 1996). The lack of
intrinsic effects in all experiments is highly indicative that cannabinoid antagonists
have no antiparkinsonian action per se. Moreover, effects of L-DOPA high doses
that approximated maximal responses in both groups of monkeys remained
unchanged by the addition of CE, which further supports the notion that CE
antiparkinsonian effects depend on modulation of dopamine responses.

Lack of antidyskinetic effects of CE may be in line with the opposite effects of the
cannabinoid agonist, nabilone, (Sieradzan et al., 2001; Fox et al., 2002). It remains
unclear where cannabinoid antagonists and agonists mediate these opposing
behavioral effects among the various locations of CB1 receptors in basal ganglia.
The status of endocannabinoid transmission after dopamine denervation remains
controversial. Changes of striatal endocannabinoids have been reported in opposite
directions (Gubellini et al., 2002; Ferrer et al., 2003). In addition, CB1 receptor
binding and mRNA were found to increase in striatum and other basal ganglia
regions (Mailleux and Vanderhaeghen, 1993; Lastres-Becker et al., 2001). However,
the precise function of this system through presynaptic and postsynaptic receptors remains obscure. CB1 receptors have modulatory effects on gabaergic, glutamatergic, and other transmitters activities that may lead to varied interaction with dopamine responses (Di Marzo et al., 1998; Gubellini et al., 2002). As a result, CB1 antagonists have been proposed to reduce bradykinesia -antiparkinsonian action-(Romero et al., 2000), but also L-DOPA-induced dyskinesias -antidyskinetic action-(Brotchie, 1998). In contrast, the activation of striatal CB1 receptors may produce motor effects by recognized mechanisms, such as the reduction of glutamate release (Gerdeeman and Lovinger, 2001; Gubellini et al., 2002) and the expression of long-term depression (Gerdeeman et al., 2002; Kreitzer and Malenka, 2007). In line with our results, Giuffrida et al. (1999) have shown reciprocal interactions between endocannabinoid and dopaminergic systems in normal conditions where CB1 blockade facilitates dopaminergic effects. Some authors (Sieradzan et al., 2001) have proposed that the effects of cannabinoid antagonists are mediated by increase of GABA reuptake in GPe through presynaptic CB1 receptors (Maneuf et al., 1996). However, the facts that CB1 receptor activation also reduces GABA release and has important actions in striatum and other regions (Herkenham et al., 1990), rather suggest the interaction of various mechanisms to produce the observed behavioral effects following systemic administration of CB1 antagonists. Data presented here do not address the mechanisms of action of CE, for which behavioral effects of intracerebral drug injections targeting regions of basal ganglia need to be tested.
In summary, results of this study indicate that the specific effects of CB1 receptor blockade by systemic administration of selective antagonists consist of enhancement of antiparkinsonian responses to dopaminergic stimulation. These effects are expressed in moderately as well as severely parkinsonian rhesus monkeys that have altered responses to dopaminergic drugs. Therefore, selective CB1 receptor antagonists may prove useful as an adjuvant to dopamine replacement therapy in patients with various disease stages. Furthermore, combined treatment with cannabinoid antagonists may help adjust L-DOPA doses to delay or reduce disabling motor complications.
Acknowledgments

We thank Jessica Whithear for her technical support in the realization of this study and the technical and veterinary staff of the Yerkes National Primate Research Center for the dedicated assistance in the care of parkinsonian monkeys. We also thank Dr. Debra Kendall (University of Connecticut) for the generous gift of the human CB1 and CB2 receptor cDNAs and cell lines, and Karen Ward and Rebecca O’Connor for their support in CE profiling techniques.
References


of nigrostriatal dopaminergic neurons increased CB1 receptor mRNA levels in

Sieradzan KA, Fox SH, Hill M, Dick JP, Crossman AR and Brotchie JM (2001)
Cannabinoids reduce levodopa-induced dyskinesia in Parkinson's disease: a pilot

excitatory neurotransmission in the substantia nigra pars reticulata.

*Neuroscience* **97**:89-97.

van der Stelt M, Fox SH, Hill M, Crossman AR, Petrosino S, Di Marzo V and
Brotchie JM (2005) A role for endocannabinoids in the generation of
parkinsonism and levodopa-induced dyskinesia in MPTP-lesioned non-human

Footnotes

This study was supported by a research grant from Pfizer, Inc., New York, NY.

1 Corresponding author

Reprint requests: Stella M. Papa, M.D. Department of Neurology, Emory University.
6000 WMRB, 101 Woodruff Circle. Atlanta, Georgia 30322, US.
E-mail: spapa@emory.edu
Legends for Figures

Figure 1. The structure of CE.

CE: 1-[7-(2-Chlorophenyl)-8-(4-chlorophenyl)-2-methylpyrazolo[1,5-a][1,3,5]triazin-4-yl]-3-ethylaminoazetidine-3-carboxylic Acid Amide Benzenesulfonate. Molecular
Formula: C24H23Cl2N7O•C6H6O3S. Molecular Weights: Free Base: 496.40; Benzenesulfonate Salt: 654.58

Figure 2. Effects of co-administration of CE with L-DOPA on motor disability scores in severely parkinsonian monkeys. A: Total motor disability scores. Each bar represents the effect of each treatment, CE V (vehicle) + LD-low, CE 0.1 mg/kg + LD-low, CE 0.3 mg/kg + LD-low, and CE 1 mg/kg + LD-low; and the same CE combinations with LD-high. Values are the average of the sum of each interval total scores during the whole antiparkinsonian response (from 30 min after treatment to the last interval of scores 50% or lower than baseline) from all monkeys. * p < 0.05 versus CE vehicle + same dose of L-DOPA. B: Time course of responses. Each curve represents the effects of each treatment combination of CE and LD-low, as described in A. Values are the average of total motor disability scores for individual intervals from all monkeys. ‘Off’ represents the baseline score taken just before drug injections (time 0), and after injections scoring starts at 30 min and follows thereafter every 20 min. ** p < 0.01 for differences in same time point between CE 0.3 mg/kg + LD-low or CE 1 mg/kg + LD-low and CE vehicle and LD-low (ANOVA for repeated measures, n=3). Data points: mean; error bars: SEM.
Figure 3. Effects of co-administration of CE with L-DOPA on motor disability scores in moderately parkinsonian monkeys. A: Total motor disability scores. Each bar represents the effect of each treatment, CE V (vehicle) + LD-low, CE 0.3 mg/kg + LD-low, and CE 1 mg/kg + LD-low; and the same CE combinations with LD-high. Values are the average of the sum of each interval total scores during the whole antiparkinsonian response (from 30 min after treatment to the last interval of scores 50% or lower than baseline) from all monkeys. ** p < 0.01 versus CE vehicle + same dose of L-DOPA. B: Time course of responses. Each curve represents the effects of each treatment combination of CE and LD-low, as described in A. Values are the average of total motor disability scores for individual intervals from all monkeys. ‘Off’ represents the baseline score taken just before drug injections (time 0), and after injections scoring starts at 30 min and follows thereafter every 20 min. Significance was not attained at individual intervals (ANOVA for repeated measures, n=3). Data points: mean; error bars: SEM.

Figure 4: Response duration with co-administration of CE and L-DOPA low dose in all monkeys. Each bar represents the effects of each treatment combination, CE V (vehicle) + LD-low, CE 0.3 mg/kg + LD-low, and CE 1 mg/kg + LD-low in individual monkeys (R0073, R1947, R1959, RG16, RLt6 and RNQ6). Values are the average of the total duration of the antiparkinsonian response (time elapsed from beginning of effects at 30 min interval to the last interval of scores 50% or lower than baseline) from 3 repeated tests for each treatment combination. * p < 0.05 versus CE vehicle.
+ LD-low in 4 out of 6 monkeys (ANOVA for repeated measures). Data points: mean; error bars: SEM.

Figure 5. Effects of co-administration of CE with L-DOPA on dyskinesias scores in severely parkinsonian monkeys. Each bar represents the effect of each treatment, CE V (vehicle) + LD-low, CE 0.1 mg/kg + LD-low, CE 0.3 mg/kg + LD-low, and CE 1 mg/kg + LD-low; and the same CE combinations with LD-high. Values are the average of dyskinesias scores for individual intervals (from 30 min to the last interval of motor disability scores 50% or lower than baseline) from all monkeys. No significant differences were found in dyskinesias scores after combination of CE with levodopa low or high doses (ANOVA for repeated measures, n=3). Data points: mean; error bars: SEM.

Figure 6. Plasma levels of L-dopa following co-administration of CE. Each curve represents the effect of each treatment, CE V (vehicle) + LD-low and CE 1 mg/kg + LD-low, on L-DOPA concentration in plasma in each of two monkeys (RGl6 and RLt6). Each data point is the average of L-DOPA concentration at a certain time from treatment administration from 3 blood samples (3 repeated experiments). Blood samples were collected at intervals that correlated with behavioral changes produced by these treatments. No significant differences were found between control and CE treatments (paired t tests). Data points: mean; error bars: SEM. Areas under the curve are: 1-L-DOPA + CE vehicle, 12,901 +/- 2,010 in RGl6, and
10,539 +/- 999 in RLt6; 2-L-DOPA + CE 1 mg/kg, 10,916 +/- 1,179 in RGl6, and 8,106 +/- 745 in RLt6 (non-significant differences).
### TABLE 1.

**Motor Disability Scores**

<table>
<thead>
<tr>
<th></th>
<th>Moderately parkinsonian Group (n=4)</th>
<th>Severely Parkinsonian Group (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balance</td>
<td>0</td>
<td>0.25 ± 0.25</td>
</tr>
<tr>
<td>Posture</td>
<td>0.25 ± 0.25</td>
<td>0.50 ± 0</td>
</tr>
<tr>
<td>Gait</td>
<td>1.63 ± 0.25</td>
<td>2 ± 0</td>
</tr>
<tr>
<td>Mobility</td>
<td>1.75 ± 0.29</td>
<td>2.17 ± 0.29</td>
</tr>
<tr>
<td>Hand Mov R/L</td>
<td>1.13 ± 0.25/1.13 ± 0.25</td>
<td>1.17 ± 0.29/1.17 ± 0.29</td>
</tr>
<tr>
<td>Leg Mov R/L</td>
<td>1.13 ± 0.25/1.13 ± 0.25</td>
<td>1.33 ± 0.29/1.33 ± 0.29</td>
</tr>
<tr>
<td>Climbing</td>
<td>1.63 ± 0.25</td>
<td>2 ± 0</td>
</tr>
<tr>
<td>Tremor</td>
<td>1.88 ± 0.25</td>
<td>2.33 ± 0.29</td>
</tr>
<tr>
<td>Food PU R/L</td>
<td>0.25 ± 0.25/0.25 ± 0.25</td>
<td>0.67 ± 0.58/1.17 ± 1.23</td>
</tr>
<tr>
<td>Social Interaction</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total MDS</td>
<td>11.88 (10-15.5)</td>
<td>16.00 (12.5-20)</td>
</tr>
</tbody>
</table>

Values correspond to the mean ± S.E.M. Total MDS is the sum of all items. Mov = movement; R/L = right/left; PU = pick up; MDS = motor disability score.
TABLE 2

Reversal by CE of the Cannabinoid Tetrad

<table>
<thead>
<tr>
<th>Dose (mg/kg s.c.)</th>
<th>Activity</th>
<th>Analgesia</th>
<th>Hypothermia</th>
<th>Catalepsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>65%</td>
<td>49%*</td>
<td>41%*</td>
<td>60%*</td>
</tr>
<tr>
<td>1.0</td>
<td>136%*</td>
<td>116%*</td>
<td>80%*</td>
<td>85%*</td>
</tr>
<tr>
<td>3</td>
<td>70%*</td>
<td>80%*</td>
<td>108%*</td>
<td>87%*</td>
</tr>
</tbody>
</table>

Data were converted to percentages with 0% being the level of activity observed after CP-55940 (CB1 agonist) alone and 100% that in the absence of CP-55940. Values are the relative level of each component after administration of CE in combination with CP-55940. * p < 0.05 vs. 0.78 mg/kg of CP-55940 alone (n = 5-7 animals per group).
### TABLE 3

Comparison of CE and L-DOPA treatments (crossover)

<table>
<thead>
<tr>
<th>Initial Treatment</th>
<th>Subsequent Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE V + LD-low*</td>
<td>A: 4.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>B: 3.5 ± 0.3</td>
</tr>
<tr>
<td>CE V + LD-high*</td>
<td>A: 2 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>B: 2.2 ± 0.05</td>
</tr>
<tr>
<td>CE 0.3 + LD V</td>
<td>C: 10.8 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>D: 10.5 ± 0.5</td>
</tr>
<tr>
<td>CE 1 + LD V</td>
<td>C: 11 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>D: 10.75 ± 0.5</td>
</tr>
</tbody>
</table>

Total scores of motor disability at 50 minutes after injection (peak effect of drugs given s.c.). Monkeys denominated with capital letters (A, B, C, D). *: p < 0.001 vs. CE 0.3 mg/kg + LD V (vehicle) or CE 1 mg/kg + LD V (vehicle). In each monkey, scores after CE 0.3 mg/kg + LD vehicle and CE 1mg/kg + LD vehicle treatments were unchanged from baseline before drug injections.
Figure 1
Figure 2

A

Motor Disability Score

LD-low  LD-high

Treatment

0  5  10  15  20  25

0  5  10  15  20

B

Motor Disability Score

CE  V + LD-low

CE 0.1 + LD-low

CE 0.3 + LD-low

CE  1 + LD-low

Time (min)

Off  30  50  70  90  110  130  150

** **
Figure 3

**A**

Motor Disability Score

<table>
<thead>
<tr>
<th>Treatment</th>
<th>V</th>
<th>0.3</th>
<th>1</th>
<th>V</th>
<th>0.3</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD-low</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LD-high</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B**

Motor Disability Score vs Time (min)

- CE V + LD-low
- CE 0.3 + LD-low
- CE 1 + LD-low

Off 30 50 70 90 110 130 150
Figure 4
Figure 5

![Bar chart showing dyskinesia scores over time for different conditions. The x-axis represents time in minutes (30, 50, 70, 90, 110, 130, 150, 170) and the y-axis represents dyskinesia score (0 to 6). Conditions include CE V + LD-low or high, CE 0.1 + LD-low or high, CE 0.3 + LD-low or high, and CE 1 + LD-low or high. The bars show variability with error bars.]