Cannabinoid CB₁ antagonists possess antiparkinsonian efficacy only in rats with very severe nigral lesion in experimental parkinsonism

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We have observed that systemic administration of cannabinoid CB₁ antagonists exerts antiparkinsonian effects in rats with very severe nigral lesion (~95% cell loss), but not in rats with less severe lesion (85–95% cell loss). Local injections into denervated striatum and corresponding globus pallidus reduced parkinsonian asymmetry. Infusions into lesioned substantia nigra enhanced motor asymmetries, corresponding globus pallidus reduced parkinsonian asymmetry.

Introduction

Brain cannabinoid CB₁ receptors are expressed in neural areas that contribute to movement such as the basal ganglia, where their density is high and they co-localize with dopamine D₂ and D₁ receptors in striatal projection neurons (Herkenham et al., 1990, 1991; Surmeier et al., 1996; Tsou et al., 1998). It is known that there is a continual intracerebral release of endogenous cannabinoid receptor agonists such as anandamide (AEA) exhibiting neuro-modulatory function (Baker et al., 2000; Giuffrida et al., 1999), and that the endocannabinoid system is an activity-dependent modulator of nigrostriatal dopaminergic neurotransmission (Beltramo et al., 2000; Cadogan et al., 1997; Glass et al., 1997; Pertwee, 1999).

Endogenous cannabinoid CB₁ activation seems to act as a brake of dopaminergic activity on dorsal striatum, and it has been proposed that it is a homeostatic counter-regulatory mechanism in the basal ganglia (Rodríguez de Fonseca et al., 1994, 1998). The interdependence between striatal CB₁ and DA receptors have led to propose that cannabinoid CB₁ ligands could be of value for improving motor deficits in degenerative diseases such as Parkinson’s disease (PD) (Conroy, 1998; Glass et al., 1997; Rodríguez de Fonseca et al., 1998). The involvement of the endocannabinoid system in Parkinson’s disease is also supported by recent findings describing up-regulation of CB₁ receptors in the striatum after dopaminergic denervation, both in animals and humans (Lastres-Becker et al., 2001; Romero et al., 2000), associated with changes in striatal anandamide (AEA) levels. In this context, either enhancement of striatal AEA levels caused by a decrease in AEA degradation (Gubellini et al., 2002), or reduction of AEA levels have been reported (Ferrer et al., 2003). Hence, the nature of the changes in anandamide levels after striatal dopaminergic denervation remains controversial.

Cannabinoid CB₁ receptor antagonists seem to be good candidates as antiparkinsonian tools. Nonetheless, blockade of CB₁ receptors has been shown to enhance dopamine D₂ receptor-mediated facilitation of motor behaviors (Giuffrida et al., 1999). Most cannabinoid antagonists available not only block endogenous CB₁-mediated effects, but they also exhibit inverse cannabimimetic effects (Pertwee, 1999), pointing to a possible efficacy of these compounds for enhancing basal dopaminergic transmission. In this regard, SR141716A has proved to be effective in ameliorating rigidity in the reserpine Parkinson’s model in rats (Di Marzo et al., 2000), but it is ineffective in MPTP-induced parkinsonian monkeys (Meschler et al., 2001), indicating that the antiparkinsonian efficacy of these compounds is also controversial.

In the present study, selective CB₁ antagonists have been tested in a rat PD model based on the unilateral destruction of nigral dopaminergic neurons. This animal model parallels human...
disorder well, and produces a hemiparkinsonian syndrome that includes motor asymmetries, sensorimotor neglect, akinesia, and forepaw use deficit (Cenci et al., 2002; Formagueria et al., 1994; Schwarting and Huston, 1996). The cannabinoid ligand N-(4-hydroxyphenyl)-arachidonamide (AM404), which is an indirect agonist of CB1 receptors through anandamide reuptake and degradation inhibition, was used to reverse the effects of CB1 antagonists in order to verify the mediation of CB1 receptors.

Materials and methods

Subjects

Male Wistar rats (275–325 g) from the breeding colony of the Faculty of Medicine of the University of Seville, Spain, were used. Laboratory temperature was kept at 22 ± 1°C, and a 12-h light–dark cycle (lights on at 08:00 h) was maintained throughout the experiment. Food (lab chow) and water were available ad libitum.

Unilateral 6-hydroxydopamine-induced nigra lesion

Thirty minutes before 6-hydroxydopamine (6-OHDA, RBI) lesion, rats were injected with the antibiotic ceftriaxone (10 mg/0.3 ml IM), and desipramine (15 mg/kg IP) in order to protect noradrenergic terminals from 6-OHDA toxicity. Rats were anesthetized with chloral hydrate (425 mg/kg IP) and placed in a Kopf stereotaxic apparatus. Saline solution (1.2 µl per site) containing 6-OHDA (5 µg/µl free base) and 0.2% ascorbic acid (Sigma, USA) was injected over 5 min with a blunted 30-gauge cannula at the following coordinates: AP = −5.2, −5.4, L = −2.2, and V = −8.2 mm (Paxinos and Watson, 1997). Control rats followed the same protocol except that the injected solution lacked 6-OHDA.

Guide cannula surgery and intracerebral injection

Anesthetized rats were given prophylactic ceftriazone and placed in a Kopf stereotaxic apparatus. A hole was drilled over the injection site, and a 22-gauge stainless-steel guide cannula (Small Parts, USA) was aimed 2 mm above the corresponding infusion site (dorsal striatum, AP = +0.5, L = ±3, and V = −5.5; external globus pallidus, AP = −0.8, L = ±2.5 and V = −6.5; substantia nigra, AP = −5.3, L = ±2.2, and V = −8.2 mm vs. bregma) (Paxinos and Watson, 1997). The guide cannula was fastened to the skull with stainless-steel screws (Small Parts, USA) and dental cement, and was fitted with a 30 gauge stainless-steel obturator, protruding 1.5 mm out of the tip of the guide cannula (Small Parts, USA). Rats were allowed to recover 48 h after surgery. Injections were performed in the home cage after removing the obturator cannula, which was replaced by a 30-gauge stainless-steel internal cannula (Small Parts, USA) connected to a Hamilton syringe and a delivery pump (Stoelting, Germany). Solutions were slowly injected over 5 min, and afterward the internal cannula was carefully removed and the obturator cannula was replaced. After completion of all the experiments, Indian ink (1 µl) was injected through the cannula, and later rats’ brains were removed and sectioned. Sections were immunostained as explained later. Cannula placement was verified under microscope, and were mapped onto a stereotaxic atlas (Paxinos and Watson, 1997), and confirmed to be in the dorsal striatum, external globus pallidum or substantia nigra.

Chemicals and doses

SR141716A (CB1 receptor antagonist) was generously gifted by Sanofi-Synthelabo Recherche (France). The compounds AM251 (CB1 antagonist), AM404 (CB1 indirect agonist) and WIN55212-2 (CB1 agonist) were provided by Tocris (Biogen, USA), and D-amphetamine and apomorphine were provided by RBI. SR141716A and AM251 were dissolved in 30%DMSO/70% distilled water. AM404 was dissolved in 30% ethanol/70% distilled water. D-amphetamine (5 mg/kg) was dissolved in saline solution (0.9% NaCl), and apomorphine (0.5 mg/kg) was dissolved in saline solution containing 0.2% ascorbic acid. Regarding DA receptor ligands, SKF 38393 (D1 DA receptor agonist), SCH 23390 (D1 DA receptor antagonist), and spiperone (D2 DA receptor antagonist) were provided by Tocris. SKF 38393 and SCH 23390 were dissolved in double-distilled water. Spiperone and quinpirole (D2 DA receptor agonist; RBI) were dissolved in 30% ethanol/70% distilled water.

SR141716A was injected at systemic doses of 0, 0.1, 0.5, and 1 mg/kg IP; and at intracerebral doses of 0, 0.5, 1, and 1.5 µg/µl. AM251 was injected at systemic doses of 0, 1, 5, and 10 mg/kg IP, and at intracerebral doses of 0, 1, 3, and 5 µg/µl. AM404 was injected at doses of 0, 1, 3, and 5 mg/kg IP. All drugs were injected at a volume of 1 ml/kg body weight, or at intracerebral volumes of 1.5 µl (intrastriatal), 1 µl (intrapallidal), and 0.5 µl (intranigral infusions). The corresponding vehicle was used for the control group in every treatment. For intrastriatal infusions of dopaminergic ligands, SCH-23390 was injected at 0, 1, and 2 µg/µl doses (free-base, 4.6 and 9.1 nmol), and SKF-38393, spiperone, and quinpirole were each administered at 0, 0.5, and 1 µg/µl doses (free-base, SKF-38393, 2.2 and 4.4 nmol; spiperone, 2.3 and 4.6 nmol; quinpirole, 2.9 and 5.8 nmol). The corresponding vehicle was used for the control group in every treatment.

Groups and general protocol

For systemic administration studies, animals belonged to two groups: (i) hemiparkinsonian rats (SR141716A, n = 24; AM251, n = 18), and (ii) sham-lesioned control rats (both ligands, n = 10). All selected parkinsonian rats presented a positive response to amphetamine >420 turns/h, as measured 15 days after lesion. On the basis of the number of turnings after amphetamine, parkinsonian rats were divided into two groups: rats with severe lesion (turning after amphetamine <1000 per hour) and rats with very severe lesion (turning >1000 per hour) (see Results for further details). One month after nigral lesion, CB1 ligands were administered every day following a “Latin-square” type design. The first 4 days served for studying amphetamine induced turning and cannabinoid ligand-induced effects (one test per day, amphetamine being injected 30 min after CB1 ligand administration). Another day served for the study of apomorphine-induced effects, and the remainder days for studying spontaneous responses after different doses of CB1 ligand injections (spontaneous tests were carried out daily in succession as follows: odor test, open-field test, and cylinder test, beginning 1 h after each CB1 receptor ligand injection). For intracerebral injections, different groups of parkinsonian and sham-lesioned rats were studied, each group with a cannula aimed at the left or right dorsal striatum, external globus pallidus, or substantia nigra. The number of rats per group comprised 5–10. One month after lesion, the CB1 antagonists SR141716A and AM251 were administered daily through the cannula at several
doses following a “Latin-square” type design, and changing the initial dose for every rat, immediately before amphetamine (one test per day). Different groups of parkinsonian rats (n = 5–9 per group) were also subjected to intrastriatal injections of dopaminergic ligands (SKF 38393, SCH 23390, spiperone and quinpirole) alone or in combination with CB1 antagonists, following a “Latin-square” type design, and changing the initial dose for every rat.

Behavioral tests and statistics

For behavioral study, we followed a methodology previously described (Espejo et al., 1998; Fornaguera et al., 1994; Marshall, 1979; Schwarting and Huston, 1996; Ungerstedt and Arbuthnott, 1970). Thus, locomotor directional bias was evaluated by quantifying either ipsiversive rotations induced by amphetamine (5 mg/kg IP) or spontaneous ipsiversive turning. The number of ipsiversive rotations was quantified from 30 to 90 min after amphetamine (only those animals observed to make more than 420 turns/h were selected, as explained). The existence of supersensitivity of striatal DA receptors was evaluated by injecting apomorphine (0.5 mg/kg IP), and the number of contralateral rotations were quantified from 5 to 65 min after injection. Net spontaneous turning was evaluated in 10-min open field test (1 × 1 m), by calculating the percentage of ipsiversive rotations. Akinisia was quantified in the open-field test through distance traveled (cm). Sensorimotor orientation was evaluated by means of the odor test, which is based on the impaired orientation of hemiparkinsonian rats to stimuli presented contralaterally (Stricker and Zigmond, 1986). A probe with the tip impregnated in ammonia was approached from the right side near the nose, and the latency for shaking the head off the probe was quantified. Forelimb asymmetry was evaluated by the cylinder test (Kirik et al., 2000), where the animal is allowed to move freely in a transparent cylinder (50 × 30 cm) until it has displayed 20 rearing postures. The numbers of left and right forepaw contacts are counted, and the data are presented as percentage of right forepaw contacts (right paw use ratio). Hemiparkinsonian rats with lesion in the left substantia nigra present a significant impairment in the contralateral (right) paw use.

Behavioral data after systemic injections was studied by two-way ANOVA (group, between variable; drug dose, within variable), followed by one-way ANOVA (drug dose as within variable) and post hoc Newman–Keuls tests. Behavioral data after intracerebral injections was analyzed by using one-way ANOVA (treatment as between variable) followed by post hoc Newman–Keuls test for comparison between groups. When variance was not homogeneous, data was logaritmically (log(x)+1) transformed prior to analysis. Student’s t tests (independent measures) were used for comparison between groups at the same dose point. Experiments were performed according the animal care guidelines of the European Communities Council (86/609/EEC).

Immunohistochemistry

Rats were killed by decapitation and brains carefully removed. Brains were postfixed and stored in 4% paraformaldehyde in phosphate buffer (PB) 0.1 M (pH 7.2–7.4) at 4°C, and immersed overnight in 25% sucrose in PBS for cryoprotection before sectioning. Coronal brain sections (30-μm thick) were cut on a cryostate and collected in PBS. Thereafter, endogenous peroxidase activity was quenched by placing sections into 0.3% H2O2 in 0.05 M Tris buffer (pH = 7.6) for 2 h. Then, sections were incubated in PBS/0.1% Triton X-100 (PBS-T) with 10% FCS (Vector, USA) and BSA (1 mg/ml, Sigma) for 4 h to block nonspecific sites. Sections were incubated overnight with mouse anti-tyrosine-hydroxylase monoclonal antibody (anti-TH, 1:1000, Sigma) in PBS-T, and after washing in PBS-T, they were incubated for 1 h with anti-mouse conjugated antibody (1:200, Chemicon, USA). Then, sections were incubated with the ABC kit (1:100, Vector, USA) for 2 h, and specifically bound antibody were revealed by using 3’-diaminobencidine tetrahydrochloride (DAB, Sigma) as chromogen, and 0.05% hydrogen peroxide (Merck). Negative control sections were incubated in the same solutions for the same incubation times as the other brain sections, with the exception that the primary antibody solution was replaced by a PBS-T solution containing 10% FCS and BSA (1 mg/ml) without the primary antibody. Sections were washed in PBS and mounted on glass slides and coverslipped with DPX.

Quantitative assessment of dopaminergic neurons in SN

The number of TH-immunoreactive neurons in the SN pars compacta was assessed by a blinded observer. Fifteen consecutive sections were used, and stained neurons in the SN were counted at 4× magnification (n = 5 per group), on the basis of Paxinos and Watson (1997) atlas. TH-positive neurons were counted when displaying a nucleus surrounded by TH-positive cytoplasm.

Agonist-stimulated [35S]GTP-γ-S binding in membranes

This study was done in search for differences in signal transduction pathways after dopaminergic degeneration in the striatum, which would help to explain cannabinoid motor effects. Cannabinoid-stimulated [35S]GTP-γ-S binding was determined as described previously (Sim et al., 1996), using 20 μg protein from membrane fractions. Membranes were incubated at 30°C for 1 h in assay buffer (50 mM Tris–HCl, 3 mM MgCl2, 0.2 mM EGTA, 100 mM NaCl, 0.1 mg/ml BSA, pH 7.4), with 10 μM of WIN 55212-2 in the presence of 20 μM GDP and 0.05 nM [35S]GTP-γ-S in a 1 ml total volume. Basal binding was measured in the absence of agonist, and nonspecific binding was measured with 10 μM guanyldimidophosphate. The reaction was terminated by rapid centrifugation (20,000 × g) at 4°C, followed by two washes with cold Tris buffer. Bound radioactivity was determined by liquid scintillation spectrophotometry, at 95% efficiency for [35S], after overnight extraction in 5 ml Ecolite scintillation fluid. Data are reported as mean ± SE values of percentage of stimulation over basal levels. Different rats were used for this experiment, comprising intact, sham, and parkinsonian animals (n = 5–10 each).

Results

Induction of hemiparkinsonism

Rats were rendered hemiparkinsonian by injecting the toxin 6-hydroxydopamine into the left substantia nigra. Those animals that showed a strong ipsiversive rotational behavior after the administration of amphetamine (>420 turns per hour), indicative of dopamine depletion in the striatum higher than 95%, were selected for the study (Fornaguera et al., 1994; Schwarting and Huston, 1996). These animals presented an overt hemiparkinsonian
correlated with nigral lesion rather than with striatal dopamine depletion (0.01). These are novel results, because number of turnings was significantly different between both groups, as shown in Table 1. The mean percentage neuronal loss in the nigral lesion, and yielding values of 85–95% DA cell loss in rats with severe lesion, and 96.9% in rats with very severe lesion (turning >98%). Following experiments, nigral neuronal loss was quantified in both groups of rats, yielding values of 85–95% DA cell loss in rats with severe lesion, and >95% cell loss in rats with very severe lesion, as shown in Table 1. The mean percentage neuronal loss in the whole substantia nigra was 90.9 ± 0.4% in rats with severe lesion and 96.9 ± 0.8% in rats with very severe lesion (significant difference between both groups, t = 4.5, P < 0.01). These are novel results, because number of turnings was correlated with nigral lesion rather than with striatal dopamine depletion in contrast to all literature studies. The findings reveal that striatal dopamine depletion higher than 95% correlates with nigral lesion higher than 85%, indicating that there is not a point-to-point relationship between nigral lesion degree and dopamine depletion in the striatum. Rats with severe lesion presented a mean basal turning response significantly lower than that of rats with very severe lesion (SR141716A, t = 2.5, P < 0.05; AM251, t = 2.1, P < 0.05), the mean turning response being <850 turns/h in rats with severe nigral lesion and >1100 turns/h in rats with very severe nigral lesion. Both groups of rats presented a similar mean dopaminergic supersensitivity, as measured through the apomorphine test (Table 1), indicating that dopaminergic supersensitivity emerged similarly regardless the lesion degree of substantia nigra.

**Agonist-stimulated \[^{35}S\]GTP-\(\gamma\)-S binding**

No differences in WIN 55,212-2-stimulated GTP-\(\gamma\)-S incorporation were detected in any group, as observed in Table 2. Hence, striatal denervation did not affect cannabinoid CB1 receptor coupling to G proteins.

**Effects of systemic administration of CB1 receptor antagonists**

Following SR141716A and AM251 treatments, two-way ANOVA indicated significant interaction (P < 0.01), dose and group effects (see legend of Fig. 2). Post hoc analyses revealed significant changes in rats with very severe nigral lesion. Thus, amphetamine-induced turning was significantly reduced after 0.1 and 0.5 mg/kg SR141716A (P < 0.01) as well as following 5 mg/kg AM251 (P < 0.01), as shown in Fig. 2. Pretreatment of these rats with AM404 (CB1 receptor indirect agonist) eliminated the capacity of SR141716A and AM251 to induce functional changes on drug-induced turning, confirming the existence of a CB1-mediated modulation of the nigrostriatal dopaminergic tone. SR141716A and AM251 were devoid of significant effects on drug-induced turnings in rats with severe nigral lesion.

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**Table 1**

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<th>Condition</th>
<th>Mean ± SEM (turns/h)</th>
<th>Range (%)</th>
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<tr>
<td>Sham</td>
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<tr>
<td>Very severe lesion</td>
<td>1102 ± 98**</td>
<td>94.4–98.9</td>
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<td>Severe lesion AM251</td>
<td>808 ± 69**</td>
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**Note:**

- **SR141716A** and AM251 were devoid of significant effects on drug-induced turnings in rats with severe nigral lesion.
- **AM251** and 0.5 mg/kg SR141716A (CB1 receptor indirect agonist) eliminated the capacity of SR141716A and AM251 to induce functional changes on drug-induced turning, confirming the existence of a CB1-mediated modulation of the nigrostriatal dopaminergic tone. SR141716A and AM251 were devoid of significant effects on drug-induced turnings in rats with severe nigral lesion.

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**Selection of parkinsonian rats with moderate and strong nigral degeneration**

Considering that turning after amphetamine in the unilateral DA depletion model is indicative of striatal dopamine depletion degree, and it parallels quite well the different stages of PD in humans, parkinsonian rats were divided a priori into two groups according to established criteria (Fornaguera et al., 1994; Schallert and Tillerson, 1999; Schwarting and Huston, 1996): rats with severe dopamine depletion (turning after amphetamine from 420 to 1000 per hour, striatal dopamine depletion >95%) and rats with very severe dopamine depletion (turning >1000 per hour, striatal dopamine depletion >98%). Following experiments, nigral neuronal loss was quantified in both groups of rats, yielding values of 85–95% DA cell loss in rats with severe lesion, and >95% cell loss in rats with very severe lesion, as shown in Table 1. The mean percentage neuronal loss in the whole substantia nigra was 90.9 ± 0.4% in rats with severe lesion and 96.9 ± 0.8% in rats with very severe lesion (significant difference between both groups, t = 4.5, P < 0.01). These are novel results, because number of turnings was correlated with nigral lesion rather than with striatal dopamine depletion in contrast to all literature studies. The findings reveal that striatal dopamine depletion higher than 95% correlates with nigral lesion higher than 85%, indicating that there is not a point-to-point relationship between nigral lesion degree and dopamine depletion in the striatum. Rats with severe lesion presented a mean basal turning response significantly lower than that of rats with very severe lesion (SR141716A, t = 2.5, P < 0.05; AM251, t = 2.1, P < 0.05), the mean turning response being <850 turns/h in rats with severe nigral lesion and >1100 turns/h in rats with very severe nigral lesion. Both groups of rats presented a similar mean dopaminergic supersensitivity, as measured through the apomorphine test (Table 1), indicating that dopaminergic supersensitivity emerged similarly regardless the lesion degree of substantia nigra.

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Table 2 WIN 55,212-2 (10 μM)-stimulated GTP-γ-S incorporation to brain membrane fraction from intact animals, animals with sham lesion in the substantia nigra, and animals lesioned with 6-hydroxydopamine (severe and very severe lesion)

<table>
<thead>
<tr>
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<tr>
<td>Left striatum</td>
<td>149 ± 18</td>
<td>163 ± 16</td>
<td>163 ± 9</td>
<td>163 ± 2</td>
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<tr>
<td>Right striatum</td>
<td>160 ± 15</td>
<td>151 ± 11</td>
<td>166 ± 12</td>
<td>175 ± 11</td>
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Left striatum corresponded to denervated one in lesioned rats. Mean ± SEM of at least five samples per group.

For spontaneous deficits following SR141716A treatment, two-way ANOVA indicated significant interaction effects for akinesia $F(4, 62) = 15.8$, $P < 0.01$, sensorimotor orientation $F(4, 62) = 8.8$, $P < 0.05$ and right forepaw use $F(4, 62) = 12.1$, $P < 0.01$. After AM251, significant interaction effects were observed for akinesia $F(4, 50) = 15.9$, $P < 0.01$ and sensorimotor orientation $F(4, 50) = 3.6$, $P < 0.05$. Post hoc analyses indicated that systemic administration of CB1 receptor antagonists improved functional deficits in rats with very severe lesion as well (Fig. 3). Thus, after SR141716A administration, significant improvements of akinesia (0.1, 0.5 mg/kg, $P < 0.05$), sensorimotor orientation (0.1 and 0.5 mg/kg, $P < 0.01$) and right forepaw use (0.5 mg/kg, $P < 0.01$) were found in the group of rats with very severe lesion, but not in the group with severe lesion (except for sensorimotor orientation, 0.5 mg/kg, $P < 0.01$).

Of note is that akinesia and sensorimotor orientation did not show significant differences with control nonparkinsonian control rats after the most effective SR141716A dose, indicating that these symptoms were strongly ameliorated by this cannabinoid CB1 antagonist. On the other hand, akinesia and sensorimotor orientation were significantly improved after AM251 (5 mg/kg, $P < 0.05$) in rats with very severe lesion, this ligand being ineffective at lower doses or in rats with severe lesion. The highest doses of both ligands (1 mg/kg for SR141716A, 10 mg/kg for AM251) frequently induced adverse side effects such as cataleptic immobility in both sham control and hemiparkinsonian rats, as shown by profound akinesia in Fig. 3.

Effects of local injections of SR141716A and AM251 into basal ganglia nuclei

In search for the neural locus of action on basal ganglia circuit of CB1 receptor antagonists, motor asymmetry in hemiparkinsonian rats was studied after in vivo infusions of SR141716A into either left (denervated) or right dorsal striata, as well as left an right external globus pallidus and substantia nigra.

Regarding left-side injections, after injections into denervated striata one-way ANOVA indicated significant dose effects for SR141716A and AM251 in rats with very severe lesion [SR141716A, $F(3, 9) = 5.6$; AM251, $F(3,12) = 2.2$, $P < 0.05$], and rats with severe lesion [SR141716A, $F(3, 9) = 4.4$; AM251, $F(3,18) = 3.7$, $P < 0.05$]. Thus, in rats with very severe lesion turning behavior after amphetamine was significantly reduced in a dose-dependent manner following 1 (P < 0.05) and 1.5 μg/μl SR141716A ($P < 0.01$) as well after 5 μg/μl AM251 ($P < 0.05$), as shown in Fig. 4.

In rats with severe lesion, turning behavior after amphetamine was significantly reduced in a dose-dependent manner following 1.5 μg/μl SR141716A ($P < 0.01$) as well after 3 and 5 μg/μl AM251 ($P < 0.05$).

Regarding intrapallidal injections, one-way ANOVA also indicated significant dose effects for SR141716A and AM251 in both groups: with very severe lesion [SR141716A, $F(3, 12) = 8.4$, $P < 0.01$; AM251, $F(3, 15) = 10.8$, $P < 0.01$] and with severe lesion [SR141716A, $F(3, 9) = 8.4$, $P < 0.01$; AM251, $F(3, 18) = 7.8$, $P < 0.01$]. Thus, in both groups of rats, turning behavior after amphetamine was significantly reduced in a dose-dependent manner following 0.5, 1, and 1.5 μg/μl SR141716A ($P < 0.01$) as well after 3 and 5 μg/μl AM251 ($P < 0.01$), as shown in Fig. 4.

Left intranigral injections were observed to induce either a reduction or no effect on amphetamine-induced turning depending on the lesion degree (Fig. 4). Thus, in rats with severe lesion one-way ANOVA indicated significant dose effects for SR141716A $F(3, 9) = 13.1$, $P < 0.01$ and AM251 $F(3, 9) = 48.7$, $P < 0.01$, because turning was significantly enhanced after 0.5 ($P < 0.05$), 1 and 1.5 μg/μl SR141716A ($P < 0.01$), as well as following 1, 3, and 5 μg/μl AM251 ($P < 0.01$). However, one-way ANOVA indicated nonsignificant dose effects in rats with very severe lesion (Fig. 4). Furthermore, the mean basal number of left turnings was significantly higher in rats without functional response after intranigral infusions than in the other group (SR141716A, $t = 5.1$, $P < 0.01$; AM251, $t = 4.3$, $P < 0.01$), further confirming that the degree of nigral degeneration was the...
variable affecting the motor effects induced by intranigral infusions of CB₁ antagonists.

After injections into nondenervated basal ganglia, the effects were found to be much lower, indicating that local CB₁ antagonism was far more effective in denervated basal ganglia. One-way ANOVA indicated significant dose effects for SR141716A after intranigral \( F(3, 15) = 6.1, P < 0.05 \) and intrastriatal \( F(3, 15) = 6.0, P < 0.05 \) infusions. Post hoc tests revealed that intrastriatal and intranigral SR141716A infusions significantly enhanced \( P < 0.05 \) the number of left turnings only after 0.5 (intranigral) and 1.5 μg/ml (intrastral). Intrastral AM251 injections significantly enhanced the number of left turnings at 5 μg/ml dose \( P < 0.05 \). Intrapallidal infusions were ineffective.

**CB₁ antagonists act modulating striatal D₁ and D₂ dopamine receptor function in opposite fashion**

In search for the functional interaction of the cannabinoid CB₁ antagonism and dopaminergic D₁ and D₂ receptors in the striatum, motor asymmetry in hemiparkinsonian rats was studied after in vivo co-infusions of SR141716A and dopaminergic ligands. Since no significant differences were observed a posteriori between rats with either very severe or severe lesion regarding dopaminergic ligand effects, data refer to as the whole group of parkinsonian rats.

As observed in Table 3, one-way ANOVA indicated significant treatment effects after local infusions of D₁ DA receptor ligands in combination with SR141716A (4.8 nmol, 1.5 μg/ml, dose with maximum effect) and AM251 (13.5 nmol, 5 μg/ml, dose with maximum effect). Thus, SCH-23390 (D₁ receptor antagonist; 4.6 and 9.1 nmol) in combination with either SR141716A or AM251 had significantly lower effects than SR141716A and SCH-23390 alone [treatment effect, \( F(4, 24) = 22.3, P < 0.01 \)] or than AM251 and SCH-23390 alone [treatment effect, \( F(4, 24) = 10.5, P < 0.01 \)]. SKF-38393 (D₁ receptor agonist, 2.2 and 4.4 nmol) in combination with either SR141716A or AM251 had significantly greater effect than SR141716A and SKF-38393 given alone [treatment effect, \( F(4, 24) = 6.2, P < 0.01 \)] or than AM251 and SKF-38393 given alone [treatment effect, \( F(4, 27) = 9.7, P < 0.01 \)]. These findings indicate that both CB₁ antagonists enhanced motor effects after stimulation of striatal D₁ DA receptors.

On the other hand, one-way ANOVA indicated significant treatment effects after local infusions of D₂ DA receptor ligands in combination with SR141716A and AM251. Thus, spiperone (D₂ receptor antagonist, 4.6 nmol) in combination with SR141716A or AM251 had significantly greater motor effects than SR141716A and spiperone alone [treatment effect, \( F(4,22) = 4.1, P < 0.05 \)] or than AM251 and spiperone alone [treatment effect, \( F(4,24) = 4.6, P < 0.05 \)]. Quinpirole (D₂ receptor agonist;
2.9 and 5.8 nmol) in combination SR141716A or AM251 had significantly lower effects than SR141716A and quinpirole alone [treatment effect, $F(4, 22) = 6.4, P < 0.01$] or than AM251 and quinpirole alone [treatment effect, $F(4, 24) = 4.1, P < 0.05$]. These findings indicate that both CB1 antagonists reduced motor effects after stimulation of striatal D2 DA receptors. Interestingly, all D1 and D2 DA receptor agonists and antagonists alone significantly reduced the number of turnings after amphetamine (see Table 3).

Local injections: histology

Only those animals where injection site was found to be correct were included for analyses. Fig. 5 illustrates the cannula tip location in left dorsal striatum, external globus pallidus and substantia nigra in hemiparkinsonian rats receiving SR141716A and AM251 infusions. Inspection of brain tissue revealed slight evidence of gliosis at the site of injection, although surrounding tissue was generally intact.

Discussion

Systemic cannabinoid CB1 antagonists exert antiparkinsonian effects only in rats with strong nigral degeneration

The data indicated that, within a dose range (0.1–0.5 mg/kg for SR141716A and around 5 mg/kg for AM251), the systemic administration of cannabinoid CB1 antagonists exerted antiparkinsonian effects in animals with very severe nigral degeneration. In this group of rats, nigral TH+ cell loss was higher than 95% of normal (analogue of last stage of human PD), and turning, akinesia, sensorimotor neglect (both ligands) and right forepaw use (SR141716A) were significantly ameliorated. In the remainder rats, characterized by less severe TH+ cell degeneration (85–95% cell loss, analogue of early to middle-stage human PD), antiparkinsonian effects were found to be mostly absent. The data showed a relationship between turning level and nigral lesion, because rats circling more than 1000/h showed a very severe nigral lesion (>95%), in contrast to lower levels of
turning. These are novel results, because all literature studies refer to striatal dopamine depletion in correlation with number of turnings. Thus, rats circling more than 420 per hour are known to present a dopamine depletion in the striatum higher than 95% (Fornaguera et al., 1994; Schwarting and Huston, 1996). However, there is not a point-to-point relation between percent nigral lesion and percent striatal dopamine depletion (Anden et al., 1966), because the data of the present study indicate that rats

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Percent change in amphetamine-induced turning after intrastriatal dopaminergic ligands and cannabinoid CB1 antagonists in parkinsonian rats</th>
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<tr>
<td><strong>SR141716A and dopamine receptor ligands</strong></td>
<td></td>
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<tr>
<td>SR</td>
<td>D1 antagonism</td>
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<td>53 ± 5**</td>
<td>SCH (4.6 nmol) + SR</td>
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<td>54 ± 5**</td>
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<td>SR</td>
<td>D2 antagonism</td>
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<tr>
<td>53 ± 5**</td>
<td>Spi (2.3 nmol) + SR</td>
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<tr>
<td>SR</td>
<td>D2 agonism</td>
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<tr>
<td>54 ± 6**</td>
<td>Qui (2.9 nmol) + SR</td>
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<tr>
<td><strong>AM251 and dopamine receptor ligands</strong></td>
<td></td>
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<tr>
<td>AM251</td>
<td>D1 antagonism</td>
</tr>
<tr>
<td>55 ± 10*</td>
<td>SCH (4.6 nmol) + AM251</td>
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<tr>
<td>AM251</td>
<td>D1 agonism</td>
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<tr>
<td>AM251</td>
<td>D2 agonism</td>
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<tr>
<td>54 ± 4**</td>
<td>Qui (2.9 nmol) + AM251</td>
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</table>

Percentage reduction of number of turnings versus vehicle-treated rats are represented. All parkinsonian rats (either with very severe or severe nigral lesion) are represented. Mean ± SEM. SR141716A was injected at 4.8 nmol (1.5 µg/µl; 1.5 µl), and AM251 was injected at 13.5 nmol (5 µg/µl; 1.5 µl). Abbreviation: SR, SR141716A; SKF, SKF38393; SCH, SCH23390; Spi, Spiperone; Qui, quinpirole.

* P < 0.05 vs. the corresponding combination of CB1 antagonist and dopaminergic ligand (lower dose).
** P < 0.01 vs. the corresponding combination of CB1 antagonist and dopaminergic ligand (lower dose).
# P < 0.05 vs. the corresponding combination of CB1 antagonist and dopaminergic ligand (higher dose).
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Percentage reduction of number of turnings versus vehicle-treated rats are represented. All parkinsonian rats (either with very severe or severe nigral lesion) are represented. Mean ± SEM. SR141716A was injected at 4.8 nmol (1.5 µg/µl; 1.5 µl), and AM251 was injected at 13.5 nmol (5 µg/µl; 1.5 µl). Abbreviation: SR, SR141716A; SKF, SKF38393; SCH, SCH23390; Spi, Spiperone; Qui, quinpirole.

* P < 0.05 vs. the corresponding combination of CB1 antagonist and dopaminergic ligand (lower dose).
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# P < 0.05 vs. the corresponding combination of CB1 antagonist and dopaminergic ligand (higher dose).
## P < 0.01 vs. the corresponding combination of CB1 antagonist and dopaminergic ligand (higher dose).

Fig. 5. Locations of infusions into (A) left dorsal striatum, (B) external globus pallidum, and (C) substantia nigra in parkinsonian rats that received either SR141716A or AM251. Rats with cannula tip locations outside the basal ganglia nucleus were discarded for statistical analysis. Plates are taken from Paxinos and Watson (1997), and they indicate millimeters from bregma.
rotating more than 420 per hour present a nigral degeneration higher than 85%, and only if rotation degree is very high (>1000 per hour), this degeneration is stronger than 95% (very severe). On the other hand, reduction in amphetamine-induced circling was not influenced by a possible hypokinetic effect of CB1 antagonists (clearly present at high doses), because akinesia was actually abolished at the most effective doses. Since the unilateral 6-OHDA-lesion model of PD in rats parallels the human disorder well (Cenci et al., 2002), these findings are remarkable because sensorimotor orientation and mostly akinesia are difficult to alleviate in human PD, and the anti-akinetic effect is highly consistent with an antiparkinsonian profile of these ligands. CB1 receptors mediated these effects because pretreatment with AM404 (CB1 indirect agonist that acts as AEA reuptake and degradation blocker) eliminated the capacity of CB1 antagonists to induce functional changes. In this regard, AM404 is also known to activate vanilloid VR1 receptors, and seems to enhance bioavailability of 2-arachidonoylglycerol, another endocannabinoid substance (Bisogno et al., 2001; Zymunt et al., 2000). These side effects could also mediate AM404-induced blocking of the antiparkinsonian efficacy of CB1 antagonists. To sum up, the results allow proposing that systemic administration of cannabinoid CB1 antagonists might be useful for treatment of advanced stage of PD in humans, where no efficacious therapies are available at present. The data support that CB1 antagonists have not a continuum of effects in parkinsonian rats, but they are only effective after advanced degeneration of substantia nigra. This fact is otherwise in line with the loss of efficacy of other drugs such as levodopa in advanced stages of PD, but indicates that the treatment could be applied to most extremely affected PD patients. However, further clinical research is needed on this topic since the findings of the present study refer to an animal model of parkinsonism and not to an actual human syndrome.

Stimulatory striatal and pallidal action, and lesion degree of substantia nigra are critical features in account of motor effects of CB1 antagonists

Regional injections of CB1 antagonists induced much stronger effects on the denervated basal ganglia circuit with respect to a nondenerverated one, in accordance with previous data with cannabinoid agonists (Sañudo-Peña et al., 1996, 1998). Intrastriatal and intrapallidal injections in the denervated circuit were observed to reliably reduce amphetamine-induced turning. Interestingly, the most effective antiparkinsonian dose of AM251 was two to three times higher than that of SR141716A, which is consistent with Kd values of both ligands (5.6 nM for SR141716A and 12 nM for AM251) (Pertwee, 1999). On the other hand, intranigral injections were found to enhance turning in rats with severe lesion, but this effect wore off after very severe nigral degeneration. These phenomena could help explain functional effects observed after systemic injections (i) systemic cannabinoid CB1 antagonists in rats with severe nigral degeneration is ineffective because the stimulatory motor effects mediated by striatal and pallidal neurons are antagonized by nigra-mediated activity, and (ii) CB1 antagonists exert antiparkinsonian effects after very severe nigral degeneration because nigra-mediated inhibition disappears. Intrastriatal effects of CB1 antagonists were similar in rats with either severe or very severe nigral lesion, further indicating that substantia nigra rather than dorsal striatum is the critical locus that account for antiparkinsonian effects of CB1 antagonists. No activation of signal transduction mechanism in the striatum after 6-OHDA-induced denervation was observed, as measured through WIN 55,212-2-stimulated GTP-γ-S incorporation, in accordance with other authors (Romero et al., 2000). This indicates that striatal dopaminergic denervation is not associated with changes in transduction pathways related to G proteins coupled to CB1 receptors, ruling out that these changes could participate on differential motor effects of CB1 antagonists.

Motor effects after infusions into denervated striatum after CB1 antagonism are mediated by a regulation in opposite fashion of D1 and D2 receptors

The observed stronger effect of SR141716A and AM251 into the denervated striatum with respect to the normal one could be also related to the known up-regulation of striatal CB1 receptors after 6-OHDA-induced lesion (Lastres-Becker et al., 2001; Romero et al., 2000). In conclusion, at the striatal level, both SR141716A and AM251 induce functional effects mostly through enhancement of motor processes mediated by D1 receptor stimulation, likely facilitated by up-regulation of CB1 receptors, leading to less disbalance between left and right basal ganglia circuits, and amelioration of spontaneous parkinsonian symptoms which are known to be dependent on the striatal dopaminergic tone (Björklund et al., 1980; Brundin et al., 1987). In this context, an increased cannabinoid CB1 receptor binding
has also been detected in patients with PD (Lastres-Becker et al., 2001).

**Systemic cannabinoid CB1 antagonists might be effective in advanced Parkinson’s disease**

The findings fit well with the proposed homeostatic counter-regulatory role of endogenous cannabinoids in basal ganglia (Rodríguez de Fonseca et al., 1994, 1998; Sañudo-Peña and Walker, 1998a; Tersigni and Rosenberg, 1996). An improvement of motor function after intrapallidal injections is also in accordance with the fact that augmented levels of endogenous cannabinoids in the globus pallidus are associated with a reduction in movement in parkinsonian rats (DiMarzo et al., 2000). Previous contradictory results after CB1 antagonists in parkinsonian rats and monkeys could be attributable to the differential efficacy of these ligands depending on the level of nigral degeneration. In support of our hypothesis, the reserpine rat model of PD is known to induce a strong DA depletion with a rapid development of striatal dopaminergic supersensitivity (within 12–24 h after reserpine) (Trugman and James, 1992). Cannabinoid CB1 antagonists are effective in ameliorating immobility in this model (DiMarzo et al., 2000). However, cannabinoid CB1 antagonists have revealed not to be effective in long-term MPTP-treated parkinsonian monkeys (Meschler et al., 2001). Other authors have shown that most severely injured parkinsonian animals subjected to chronic MPTP regimen present 70–80% nigral TH+ cell loss and >95% of striatal DA depletion (DiMonte et al., 2000). According to the present study, the percentage surviving population of nigral TH+ neurons is a critical factor regarding the antiparkinsonian nature of cannabinoid CB1 antagonists, and its systemic administration appears not to exert functional effects when nigral loss of dopaminergic neurons is lower than 95%.

**General discussion**

The present study provides evidence that cannabinoid CB1 antagonists might be of value as therapeutic tools in advanced human PD, within a dose range. CB1 antagonists have not a continuum of effects in parkinsonian rats, but they are effective after advanced degeneration of substantia nigra. These ligands were ineffective or induced catalepsy at lower or higher doses, respectively, which suggests the existence of a “therapeutic window” for these compounds as potentially antiparkinsonian tools. Another advantage of cannabinoid CB1 antagonists is that, unlike CB1 agonists, they are devoid of psychoactive effects, thereby further enhancing their potential therapeutic benefit. This work should stimulate research on the clinical applicability of cannabinoid CB1 antagonists in severe PD, taking into account that, as explained, the findings of the present study refer to an animal model of parkinsonism and not to an actual human syndrome.

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**References**


Gubellini, P., Picconi, B., Barti, M., Battista, N., Calabresi, P., Centonze, D.,
